FINAL REPORT

Study Title

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY

Test Substances

AQ, AR, AS, AT
AU, AW, AY

Reference Substance

AV

Authors

John W. Harbell, Ph.D.
Allison Hilberer, B.A.

Study Completion Date

December 13, 2005

Performing Laboratory

Institute for In Vitro Sciences, Inc.
21 Firstfield Road, Suite 220
Gaithersburg, MD 20878

Study Number

05AD93-AE00.350064

Laboratory Project Number

4137

TABLE OF CONTENTS

STATEMENT OF COMPLIANCE

The Bovine Corneal Opacity And Permeability Assay with Two Time Exposures and Optional Histology of the test substances, AQ, AR, AS, AT, AU, AV (reference substance), AW, and AY, was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

> The identity, strength, purity and composition or other characteristics to define the test substances have not been determined by the testing facility.

John W. Harbell, Ph.D. Date
Study Director

QUALITY ASSURANCE STATEMENT

Study Title: Bovine Corneal Opacity and Permeability Assay with Two Time Exposures and Optional Histology

Study Number: 05AD93-AE00.350064

Study Director: John W. Harbell, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLP Standards (40 CFR 792 and 40 CFR 160) and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected and report dates of QA inspections of this study:

Phase Inspected	Audit Date(s)	Reported to Study Director	Reported to Management
Protocol and Initial Paperwork	13-Jul-05	13-Jul-05	13-Jul-05
Permeability Measurement	13-Jul-05	19-Jul-05	25-Jul-05
PAI – Microtomy	09-Aug-05	30-Aug-05	31-Aug-05
PAI – Individual Animal Data and Supporting Documentation	24-Aug-05	30-Aug-05	31-Aug-05
Histology Evaluation – Negative Control and 05AD98 3min exposure	14-Nov-05	21-Nov-05	21-Nov-05
Final Report and Data	12-Dec-05	12-Dec-05	13-Dec-05

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

_____ _____

Amanda K. Ulrey, RQAP-GLP Date
Quality Assurance

SIGNATURE PAGE

Initiation Date:	July 12, 2005
Completion Date:	December 13, 2005
Sponsor:	
Sponsor's Representative:	
Testing Facility:	Institute for In Vitro Sciences, Inc. 21 Firstfield Road, Suite 220 Gaithersburg, MD 20878
Archive Location:	Institute for In Vitro Sciences, Inc. Gaithersburg, MD 20878
Study Director:	_____ John W. Harbell, Ph.D. Date
Laboratory Management:	Greg Mun, B.A.
Principle Investigator (slide preparation): Pathology Associates, A Charles River Company	David A. Hodge HT (ASCP)
Histological Evaluation performed by:	John W. Harbell, Ph.D.

TEST SUBSTANCE AND REFERENCE SUBSTANCE RECEIPT

IIVS Test Substance Number	Sponsor's Designation	Physical Description	Receipt Date	Storage Conditions*
05AD93	AQ	clear light yellow semi-viscous liquid	6/23/05	room temperature
05AD94	AR	clear light yellow semi-viscous liquid	6/23/05	room temperature
05AD95	AS	clear light yellow semi-viscous liquid	6/23/05	room temperature
05AD96	AT	clear light yellow non-viscous liquid	6/23/05	room temperature
05AD97	AU	clear light yellow non-viscous liquid	6/23/05	room temperature
05AD98	AV (reference substance)	clear light yellow non-viscous liquid	6/23/05	room temperature
05AD99	AW	clear light yellow non-viscous liquid	6/23/05	room temperature
05AE00	AY	clear light yellow non-viscous liquid	6/23/05	room temperature

* - Protected from exposure to light

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY

INTRODUCTION

The Bovine Corneal Opacity and Permeability Assay (BCOP) was used to assess the potential ocular irritancy of the test substances to isolated bovine corneas. Bovine corneas, obtained as a by-product from freshly slaughtered animals, were mounted in special holders and exposed to the test substances. An *in vitro* score was determined for each of two exposure times tested for each test substance based on the induction of opacity and permeability (to fluorescein) in the isolated bovine corneas.

The purpose of this study was to evaluate the potential ocular irritancy of the test substances as measured by changes in opacity and permeability (to fluorescein) in isolated bovine corneas. The laboratory phase of this study was conducted from July 13, 2005 to November 17, 2005 at the Institute for In Vitro Sciences, Inc. Three corneas were treated with each test substance at two exposure times of 3 and 10 minutes. Based on changes in corneal opacity and permeability (relative to the control corneas), an *in vitro* score was determined at each exposure time.

MATERIALS AND METHODS

Bovine Eyes

Bovine eyes were obtained from a local abattoir as a by-product from freshly slaughtered animals (J.W. TREUTH & SONS, Inc., Baltimore, MD). The eyes were excised and then placed in Hanks' Balanced Salt Solution, containing Penicillin/Streptomycin (HBSS), and transported to the laboratory on ice packs. Immediately upon receipt of the eyes into the laboratory, preparation of the corneas was initiated.

Preparation of Corneas

The eyes were grossly examined for damage and those exhibiting defects were discarded. The tissue surrounding the eyeball was carefully pulled away and the cornea was excised such that a 2 to 3 mm rim of sclera was present around the cornea. The isolated corneas were then stored in a petri dish containing HBSS until they were mounted in a corneal holder. The corneas were mounted in the holders with the endothelial side against the O-ring of the posterior chamber. The anterior chamber was then positioned on top of the cornea and the screws were tightened. Starting with the posterior chamber, the two chambers were then filled with Minimum Essential Medium (EMEM) without phenol red, containing 1% fetal bovine serum and 2 mM L-glutamine (Complete MEM). Each corneal holder was uniquely identified with a number written in permanent marker, on both the anterior and posterior chambers. The corneal holders were incubated at 32 ± 1ºC for a minimum of 1 hour.

Controls

The positive control used in this study was neat ethanol (Pharmco). The negative control used in this study was sterile, deionized water (Quality Biological).

Test Substance Preparation

As instructed by the Sponsor, each test substance was administered to the test system without dilution.

Test Substance pH Determination

The pH of each test substance was determined using pH paper (EMD Chemicals Inc.). Initially, each test substance was added to 0-14 pH paper with 1.0 pH unit increments to approximate a narrow pH range. Next, each test substance was added to 7.5-14 pH paper (EM Science) with 0.5 pH unit increments, to obtain a more accurate pH value. The pH values obtained from the narrower range pH paper are presented in Table 1.

Bovine Corneal Opacity and Permeability Assay

After a minimum of 1 hour of incubation, the corneas were removed from the incubator. The medium was removed from both chambers and replaced with fresh Complete MEM. The initial opacity was determined for each cornea using a Spectro Designs OP-KIT opacitometer. Three corneas, whose initial opacity readings were close to the median opacity for all the corneas, were selected as the negative control corneas. The treatment of each cornea was identified with the test substance number written in permanent marker on colored tape, affixed to

each holder. The medium was then removed from the anterior chamber and replaced with the test substance, positive control, or negative control.

Method for Testing Liquid or Surfactant Materials

The liquid test substances, AQ, AR, AS, AT, AU, AV, AW, and AY, were tested neat. An aliquot of 750 µL of the test substance, positive control, or negative control was introduced into the anterior chamber while slightly rotating the holder to ensure uniform distribution over the cornea. One group of three corneas was incubated in the presence of each test substance at 32 ± 1ºC for 3 minutes. A second group of three corneas was incubated in the presence of each test substance at 32 ± 1ºC for 10 minutes. Each test substance was tested in at least one valid assay. The reference substance, AV, was tested in additional assays for comparison.

Test substances, AQ, AR, AS, AT, AU, and AV, were tested in the assay performed on July 13, 2005. The test substances were tested as described above. The positive control was tested in three corneas at 32 ± 1ºC for 10 minutes. The negative control was tested in three corneas at 32 ± 1ºC for 10 minutes. After the 3 and 10-minute exposure times, the control or test substance treatments were removed. The epithelial side of the corneas was washed at least three times with Complete MEM (containing phenol red) to ensure total removal of the control or test substances. The corneas were then given a final rinse with Complete MEM (without phenol red). The anterior chamber was refilled with fresh Complete MEM and an opacity measurement was performed. The corneas were returned to the incubator for approximately 2 hours after which a final measure of opacity was obtained.

Test substances, AV, AW, and AY, were tested in the assays performed on July 14, 2005 and July 18, 2005. The positive control in vitro score was not within the acceptable range on July 14 and so the assay was repeated. The test substances were tested as described above. The positive control was tested in three corneas at 32 ± 1ºC for 10 minutes. The negative control was tested in three corneas at 32 ± 1ºC for 60 minutes. After the 3, 10, and 60-minute exposure times, the control or test substance treatments were removed. The epithelial side of the corneas was washed at least three times with Complete MEM (containing phenol red) to ensure total removal of the control or test substances. The corneas were then given a final rinse with Complete MEM (without phenol red). The anterior chamber was refilled with fresh Complete MEM and an opacity measurement was performed. The corneas exposed to the test substances and the positive control (3 and 10 minute exposure) were returned to the incubator for approximately 2 hours after which a final measure of opacity was obtained. The corneas exposed to the negative control (60 minute exposure) were returned to the incubator for approximately 1 hour after which a final measure of opacity was obtained. Only the results of the assay performed on July 18, 2005 for the test substances, AV, AW, and AY, with acceptable positive control results, were considered valid and are presented in this report.

After the final opacity measurement was performed, the medium was removed from both chambers of the holder. The posterior chamber was filled with fresh Complete MEM and 1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber. The corneas were then incubated in a horizontal position (anterior side up) for approximately 90 minutes at 32 ± 1ºC. At the end of the 90-minute incubation period, the medium was removed from the posterior chamber and placed into tubes numbered corresponding to chamber number. Aliquots of 360 µL from the numbered tubes were placed into their designated wells on a 96-well plate. The optical density at 490 nm (OD_{490}) was determined using a Molecular Devices *V*max kinetic microplate reader. If the OD_{490} value of a control or test substance sample was 1.500 or above, a 1:5 dilution of the sample was prepared in Complete MEM (to bring the OD_{490} value within the linear range of the platereader). A 360 µL sample of each 1:5 dilution was transferred to its specified well on the 96-well plate. The plate was read again and the final reading was saved to a designated print file.

Fixation of Corneas

After the medium was removed for the permeability determination, each cornea was carefully separated from its corneal holder and transferred to an individual prelabeled tissue cassette containing a biopsy sponge. The endothelial surface of each cornea was placed on the sponge to protect it. The cassettes were placed in 10% neutral buffered formalin to fix the corneal tissue for at least 24 hours. The fixed corneas will be stored up to one year.

Histological Evaluation

As instructed by the Sponsor, a histological evaluation was performed on AV, and AW collected from the assay performed on July 18, 2005. The fixed corneas were transferred to Pathology Associates, A Charles River Company (Frederick, MD) for embedding, sectioning, and staining. Each cornea was paraffin-embedded, bisected, and the two halves mounted in the paraffin block so that a section of each half could be cut and placed on a single slide. Each slide was then stained with hematoxylin and eosin. Slides were returned to IIVS for evaluation.

Presentation of Data

Opacity Measurement: The change in opacity for each cornea (including the negative control corneas) was calculated by subtracting the initial opacity reading from the final opacity reading. These values were then corrected by subtracting from each the average change in opacity observed for the negative control corneas. The mean opacity value of each treatment group was calculated by averaging the corrected opacity values of each cornea for that treatment condition.

Permeability Measurement: The mean OD_{490} for the blank wells was calculated. The mean blank OD_{490} was then subtracted from the raw OD_{490} of each well (corrected OD_{490}). Any dilutions that were made to bring the OD_{490} readings into the linear range of the platereader (OD_{490} should be less than 1.500), had each diluted OD_{490} reading multiplied by the dilution factor. The final corrected OD_{490} of the test substances and the positive control was then calculated by subtracting the average corrected OD_{490} of the negative control corneas from the corrected OD_{490} value of each treated cornea:

Final Corrected OD_{490} = (raw OD_{490} – mean blank OD_{490}) – average corrected negative control OD_{490}

The mean OD_{490} value of each treatment group was calculated by averaging the final corrected OD_{490} values of the treated corneas for that treatment condition.

The following formula was used to determine the *in vitro* score:

In Vitro Score = Mean Opacity Value + (15 x Mean OD_{490} Value)

Criteria for Determination of a Valid Test

The BCOP assay was accepted when the positive control (ethanol) caused an *in vitro* score that fell within two standard deviations of the historical mean.

RESULTS AND DISCUSSION

Bovine Corneal Opacity and Permeability Assay

Table 1 summarizes the opacity, permeability, and *in vitro* score for each test substance. Table 2 summarizes the opacity, permeability, and *in vitro* score for the positive control. Since the results of the positive control fell within two standard deviations of the historical mean (within a range of 40.1 to 65.0), the assays were considered valid. The opacity and permeability data for the individual corneas may be found in Appendix B.

Table 1
BCOP Results of the Test Substances

Assay Date	IIVS Test Substance Number	Sponsor's Designation	Conc.	Exposure Time	Mean Opacity Value	Mean OD_{490} Value	In Vitro Score	pH
7/13/05	05AD93	AQ	Neat	3 minutes	7.3	2.700	47.8	13.0
				10 minutes	7.0	5.190	84.9	
	05AD94	AR	Neat	3 minutes	27.3	2.987	72.1	14.0
				10 minutes	47.3	4.582	116.1	
	05AD95	AS	Neat	3 minutes	5.7	2.060	36.6	13.0
				10 minutes	5.7	4.944	79.8	
	05AD96	AT	Neat	3 minutes	6.3	2.899	49.8	14.0
				10 minutes	3.0	5.504	85.6	
	05AD97	AU	Neat	3 minutes	20.0	2.969	64.5	14.0
				10 minutes	52.0	4.677	122.2	
	05AD98	AV	Neat	3 minutes	26.7	2.807	68.8	14.0
				10 minutes	100.7	6.079	191.8	
7/18/05	05AD98*	AV	Neat	3 minutes	33.0	3.542	86.1	14.0
				10 minutes	96.0	5.689	181.3	
	05AD99	AW	Neat	3 minutes	4.0	1.709	29.6	14.0
				10 minutes	2.7	2.695	43.1	
	05AE00	AY	Neat	3 minutes	43.3	2.424	79.7	14.0
				10 minutes	119.7	4.977	194.3	

* - Reference Substance, tested in each assay

Table 2
BCOP Results of the Positive Control

Assay Date	Positive Control	Exposure Time	Mean Opacity Value	Mean OD_{490} Value	*In Vitro* Score
7/13/05	Ethanol	10 minutes	26.7	0.970	41.2
7/18/05	Ethanol	10 minutes	31.3	1.197	49.3

Cornea exposed to two test substances were selected by the Sponsor for histological evaluation. The results of that evaluation are presented below.

Histological Evaluation

Basis for Histological Evaluation

Damage to the epithelial layer is generally the first change seen in the cornea. This is not surprising as the test materials are applied topically to this "unprotected" epithelium. Each "layer" of the epithelium is scored for cell loss or damage. Figure 1 shows the structure of the epithelium from a control cornea (not from this study). Changes to the surface epithelium (squamous and upper wing cell epithelium) in the BCOP assay are usually not predictive of lasting corneal changes *in vivo*. The loss of the squamous and upper wing cell layers in the excised bovine cornea by surfactant-based test substances appears to coincide with mild to moderate damage to the conjunctiva of the rabbit *in vivo*. This type of damage is typically reversible in the rabbit. Many types of surfactants (e.g., sodium lauryl sulfate) are expected to lyse these cells so that they are progressively lost from the epithelial surface. The positive control, ethanol, provides an example of a different type of damage. In this case, the surface squamous epithelial cells are coagulated and fixed in place. The coagulated tissue stains heavily with eosin (see Figure 5). In addition, the wing and basal cells are vacuolated and the adhesion between the basal cells and the basal lamina is disrupted. Such changes can lead to sloughing of the epithelium. Lesions in the deep wing cell and basal cell layers, either by cell lysis or coagulation, are associated with more damage *in vivo*. Much of the available data come from studies on surfactants and show that loss of the bovine corneal epithelium is predictive of some corneal opacity in the rabbit[1]

Special effort has been made to detect changes in the stromal elements of the corneas. Jester[2], Maurer[3,4] and others have shown for a range of chemical classes that depth of injury in

[1] Gettings, SD, Lordo, RA, Hintze, KL, Bagley, DM, Casterton, PL, Chudkowski, M, Curren, RD, Demetrulias, JL, DiPasquale, LC, Earl, LK, Feder, PI, Galli, CL, Glaza, SM, Gordon, VC, Janus, J, Kuntz, PJ, Marenus, KD, Moral, J, Pape, WJW, Renskers, KJ, Rheins, LA, Roddy, MT, Rozen, MG, Tedeschi, JP, and Zyracki, J. (1996) The CTFA evaluation of alternatives program: an evaluation of *in vitro* alternatives to the Draize primary eye irritation test. (Phase III) Surfactant-based formulations. **Food Chemical Toxicology** 34:79-117.

[2] Jester, J.V., Li, H.F., Petroll, W.M., Parker, R.D., Cavanaugh, H.D., Carr, G.J., Smith, B., and Maurer, J.K. (1998) Area and depth of surfactant-induced corneal injury correlates with cell death. **Invest Ophthalmol Vis Sci** 39:922-936.

[3] Maurer, J.K. and Parker, R.D. (1996) Light microscopic comparison of surfactant-induced eye irritation in rabbits and rats at three hours and recovery/day 35. **Toxicologic Pathology** 24:403-411.

the early hours after exposure can be predictive of the eventual degree and duration of the ocular lesions. Epithelial damage alone, in the rabbit cornea, is associated with expected recovery provided the basal lamina is intact. Deep injury to the stroma has more serious consequences. Stromal damage may be manifest by several types of changes within the tissue. These may include swelling within the stromal collagen matrix and loss/damage to the keratocytes. These changes do not necessarily occur together. Stromal swelling may be detected by the presence of vacuole-like "holes" in the organized collagen matrix. Their appearance suggests that liquid has entered the matrix expanding space between the fibers. Examples of these vacuoles may be seen in Figure 8 where the positive control exposure has induced some stromal swelling. The depth and degree of vacuolization can be indicative of the degree of injury to the cornea and/or penetration of the test substance into the tissue. Loss of the effective epithelial or endothelial barrier will allow water (medium) to enter the stroma and produce the collagen matrix vacuolization (swelling). Viable epithelium or endothelium will not only retard entry of the water but actively transport it out of the stroma. As the viability of epithelium declines, both the barrier and transport capacities are reduced. Thus, the amount of water accumulating in the upper stroma reflects the damage to the epithelium. When the full epithelial layer is lost over a section of stroma, stromal swelling can increase the stromal thickness by 50% or more over the control corneal stroma. Such changes are often observed when corneas treated with severe irritants are incubated for extended periods after exposure. Loss of the endothelium can also lead to appreciable deep stromal swelling. The loss may result from test substance penetration or mechanical damage. Mechanical damage tends to be more focal (patchy) in nature while the true toxic response to the test substance exposure tends to extend across much of the cornea. When focal (mechanical) damage is present, the majority of the collagen matrix vacuolization will be located in the deep stroma (just above Descemet's Membrane). In contrast, test substance-induced toxicity to the endothelium should include both epithelial as well as endothelial damage so that collagen matrix vacuolization will be observed throughout the stroma. Such extreme damage may increase the stromal thickness two fold over the control corneal stroma thickness.

In vivo, the inflammatory response that follows deep initial injury can, itself, lead to permanent lesions through "scar" collagen deposition or neovascularization in the corneal stroma. The authors cited above have suggested that the damaged keratocytes are involved in initiating this inflammatory process. Classic inflammatory changes (e.g., inflammatory cell infiltration) are possible only in the presence of active circulation through the limbus. Since the isolated cornea has no source of inflammatory cells, the potential for test substance-induced inflammation is judged by the changes in the extracellular matrix and particularly the keratocytes. Some forms of damage are more easily recognized than are others. Necrotic cell death, as might follow exposure to a strong alkaline, would be quite apparent since the cellular components rapidly break down. More subtle damage could also lead to a delayed cell death and release of inflammatory mediators. Nuclear changes (vacuolization [swelling], punctate chromatin condensation, pyknosis or karyorrhexis) are signs of this process. Cytoplasmic changes can also be informative. Vacuole formation and/or loss of basic elements (mRNA for example) are also indicative of the beginning of the degenerative process. The cell cytoplasm normally stains with both basophilic (hematoxylin) and acidophilic (eosin) stains. When the basic elements are lost, eosinophilic staining predominates. This type of change is reported as cytoplasmic eosinophilia. Harbell and Curren[5] have reported that mechanical removal of the corneal

---

[4] Maurer, J.K., Parker, R.D., and Jester, J.V. (2002) Extent of initial corneal injury as the mechanistic basis for ocular irritation: key findings and recommendations for the development of alternative assays. **Regulatory Toxicology and Pharmacology** 36:106-117.

[5] Harbell, J.W. and Curren, R.D. (2005) Using histological evaluation to enhance the Bovine Corneal Opacity and Permeability (BCOP) assay. ALTEX 42(Special Issue):236.

epithelium leads to marked stromal swelling (marked collagen matrix vacuolization). The keratocytes in the zone of marked swelling undergo changes as well. The nuclei become enlarged and the cytoplasmic staining is very eosinophilic. Thus, when test substance-treated corneas show only this type of keratocyte change (with stromal swelling), the change may be the result of the stromal swelling rather than direct action of the test substance on the cells.

Since depth of injury (both extracellular and cellular) to the stroma is important in the analysis, a means to describe the depth of injury is necessary as part of this analysis. Determining this depth is not always a straightforward process. Because of the topical application of the test substance to the epithelium, one would expect that exposure to the stroma would progress from the area just under Bowman's Layer down through the stroma to Descemet's Membrane. There is no external inflammatory process *in vitro*, so one might also expect the progression of damage to follow the progression of exposure. That means that damage to the stroma should first appear close to Bowman's Layer. As the damage to the stroma increases, deeper layers might be involved. This implies that one would want to express damage as progressing from the anterior (Bowman's Layer) to the posterior (Descemet's Membrane) and express it as a fraction of the total stromal depth involved. However, collagen matrix vacuolization can (and often does) increase total stromal thickness. Thus, measurements of the depth of a stromal lesion can be complicated by the change in overall stromal thickness. To account for stromal swelling, this depth is actually estimated from the percentage of the stromal cross section that remained undamaged (starting at the posterior border). For example, a cornea reported to show collagen matrix vacuolization to 30% depth would mean that 70% of the cross section of that cornea (starting at Descemet's Membrane) did not show vacuolization. For this report, depth of stromal damage is reported as the percentage of the normal corneal depth (cross-section) involved, starting from the anterior border (Bowman's Layer). It should be clearly understood that the percentage of the stromal depth is only an estimate developed by evaluating several fields in each cornea (where possible). The values are, by necessity, approximations of an average depth to which the lesion extended (e.g., collagen matrix vacuolization). It would be unwise to try to compare small differences in the reported depth. Rather, one should focus on broader bands of depth (e.g., upper, middle and deep stroma).

Photomicrographs of the epithelium and stroma are intended to illustrate the degree of damage at the indicated depth. Where little or no damage to the stroma was detected, the area directly under Bowman's Layer was photographed since that is where one would expect to see damage if it had occurred. Images were prepared using a Spot Insight Digital Camera and Spot 4.0.8 software (Diagnostic Instruments, Inc., Sterling Heights, MI). The color balance of the images was corrected to better represent the colors that would be seen through the microscope.

With the digital camera and associated software, it is possible to measure distances within the captured image. This feature can be used to measure corneal thickness directly. Such measurements require a true cross-section of the cornea (i.e., a perpendicular section relative to the corneal surface plane). In some cases, a true cross-section is not available. The Descemet's Membrane thickness is used to estimate how close the section is to a true cross section. The appearance of an unusually thick membrane suggests that the section was cut at a tangent to the true cross section (or a very old animal). If the measured corneal section did not appear to be a true cross section, the observation was noted. Even in a true cross section, the isolated cornea is not of uniform thickness but rather shows peaks and valleys. Therefore, an effort has been made to select "representative" cross-sections that are neither extreme "peaks" nor "valleys" for measurement of corneal thickness. An example of such a measurement is seen in Figure 4. The values obtained should be considered "representative" of the treatment group rather than strict

quantitative measures. Many more measurements would be required to provide a quantitative comparison. In some cases, the treated corneas will have lost most of their epithelium or it has been compromised (i.e., ethanol treatment). In those cases, the stromal thickness of the treated corneas should be compared to the stromal thickness of the negative control-treated corneas.

Histological Evaluation

The negative control corneas were treated for 10 minutes with sterile, deionized water (slides B7930 – B7932). The negative control-treated epithelium was composed of three layers. The basal cell layer was a well-formed, columnar-cell region directly attached to the Bowman's Layer. The basal cells were always tightly attached to each other. Several layers of wing cells covered the columnar basal layer. In both of these layers, the cell nuclei showed diffuse chromatin without clear nucleoli. Rare mitotic figures were seen in the basal layer. The squamous layer was flattened with limited cytoplasm and highly condensed nuclei (Figure 2).

The stromal elements showed well-organized collagen matrix fibers with dispersed keratocytes. Keratocyte nuclei showed a range of morphologies from moderate sized (smaller than the epithelial nuclei) with diffuse basophilic staining to narrow, elongated and condensed with dark basophilic staining. Cytoplasmic staining, when it was visible, was moderately basophilic. Rare cells, with eosinophilic cytoplasmic staining, were observed. Collagen bundles were generally parallel and well ordered (Stroma just under Bowman's Layer, Figure 3).

The Descemet's Membrane was prominent. The endothelial layer could be seen in most sections and was reasonably well maintained.

A cross section of the negative control showing the general thickness of the whole cornea and stroma is provided in Figure 4.

The positive control corneas (slides B7933 – B7935), treated for 10 minutes with 100% ethanol, showed the classic pattern of squamous layer coagulation (darkening) and marked vacuolization in the wing and basal cell nuclei (Figure 5). The loss of adhesion between the basal cells (cell to cell) and the basal lamina was marked. The epithelium was probably not viable at the time of fixation. Overall, the positive control-treated corneas were thicker than the negative control-treated corneas (Figure 6). In the stroma directly below Bowman’s Layer, the collagen matrix showed slight hypereosinophilic staining suggestive of some coagulation. Below this zone, moderate collagen matrix vacuolization extended past 50% depth. In the upper stroma, there was a decrease in the density of viable keratocytes (Figure 7) as reflected by a marked increase in the frequency of keratocytes showing nuclear vacuolization and abnormal chromatin condensation (Figure 8). In the stroma below mid depth, the keratocytes showed a moderate increase in the frequency of cells with enlarged nuclei and cytoplasmic eosinophilia (Figure 9). The endothelial cells were generally intact (similar to the negative control-treated corneas).

Table 3
Histological Observations on the Test Substance -Treated Corneas

IIVS Number	Sponsor's Designation	Observations	Figure #
05AD98 Slides B7936 – B7938	AV, neat, 3-minute exposure, 120-minute post-exposure, 07/18/05	Epithelium: The epithelium was completely lost from all sections (Figure 10). Bowman's Layer was visible. Stroma: The test substance-treated corneas were thicker than the negative control-treated corneas (Figure 11). Moderate collagen matrix vacuolization was observed through most of the stroma. Given the complete loss of epithelium, the degree of collagen matrix vacuolization was much less than expected. In the upper half to two thirds of the stroma, the keratocytes showed a marked increase in the frequency of cells with nuclear granularization (Figure 12). In the deeper stroma (above Descemet's Membrane), the keratocytes showed a moderate increase in the frequency of cells with enlarged nuclei and cytoplasmic eosinophilia (Figure 13). The deep stroma did not show increased vacuolization. Endothelium: The endothelium was clearly intact in two of the three corneas.	10 - 13
05AD98 Slides B7939 – B7941	AV, neat, 10-minute exposure, 120-minute post-exposure, 07/18/05	Epithelium: The epithelium was completely lost as was Bowman's Layer (Figure 14). Stroma: The test substance-treated corneas were thicker than the negative control-treated corneas (Figure 15). However, the degree of swelling was not nearly what would be expected with the complete loss of the epithelium and endothelium. Much of the extracellular collagen may have been denatured or the hydroscopic components of the matrix lost. Moderate vacuolization extended past mid depth. In this zone, the keratocytes showed nuclear granularization and loss of basophilic staining (Figure 16). The keratocytes were probably not viable in the treated corneas. In the deep stroma, there was marked vacuolization above Descemet's Membrane reflecting the loss of endothelium (Figure 17). Endothelium: The endothelium was lost or nonfunctional in all sections.	14 - 17
05AD99 Slides B7942 – B7944	AW, neat, 3-minute exposure, 120-minute post-	Epithelium: The epithelium above the basal layer was lost and the basal layer damaged in most fields. In a minority of fields, patches of basal cells remained. However, Bowman's Layer was	18 - 21

IIVS Number	Sponsor's Designation	Observations	Figure #
	exposure, 07/18/05	intact in all sections (Figure 18). Stroma: The test substance-treated corneas were thicker than the negative control-treated corneas (Figure 19). Marked collagen matrix vacuolization extended through the upper third of the stroma and moderate vacuolization extended past mid depth. This vacuolization would be expected from the loss of epithelial integrity and the resulting influx of water. No indication of protein denaturation was observed. In the upper third of the stroma, there was a marked to moderate increase in the frequency of keratocytes with nuclear condensation and cytoplasmic eosinophilia (Figure 20). In the stroma around mid depth, the keratocytes showed some nuclear enlargement and cytoplasmic eosinophilia (Figure 21). In the deep stroma, the keratocytes were mostly normal but with some increase in cytoplasmic eosinophilia. Endothelium: The endothelium was generally intact and there was an absence of vacuolization above Descemet's Membrane suggesting that the endothelium was functional.	
05AD99 Slides B7945 – B7947	AW, neat, 10-minute exposure, 120-minute post-exposure, 07/18/05	Epithelium: The epithelial cells were lost or severely damaged in corneas #20 and #22 (Figure 22). Slightly more epithelium remained on cornea #23 (consistent with the relative fluorescein passage through this cornea)(Figure 23). Bowman's Layer was intact in all sections. Stroma: The test substance-treated corneas were thicker than the negative control-treated corneas (Figure 24). Marked collagen matrix vacuolization extended through the upper third of the stroma and moderate vacuolization extended past mid depth. This vacuolization would be expected from the loss of epithelial integrity and the resulting influx of water. No indication of protein denaturation was observed. In the upper half of the stroma, there was a marked to moderate increase in the frequency of keratocytes with nuclear condensation and cytoplasmic eosinophilia (Figure 25). In the deep stroma, the keratocytes showed some nuclear enlargement and cytoplasmic eosinophilia (Figure 26). Endothelium: The endothelium was generally intact and there was an absence of vacuolization	22 - 26

IIVS Number	Sponsor's Designation	Observations	Figure #
		above Descemet's Membrane suggesting that the endothelium was functional.	

The figures displayed on the subsequent pages of this report are representative hematoxylin and eosin-stained cross-sections presented at the indicated magnification. The black bar, on each micrograph, represents 100 µm. Arrows from the text to the figures are intended to show examples of the lesions mentioned. Not all lesions are marked. The vertical double-headed arrows mark the limits of the epithelium in the appropriate figures.

Figure 1. An example of a control cornea showing the three layers of the epithelium, Bowman's Layer and the upper stroma (magnification 290x)

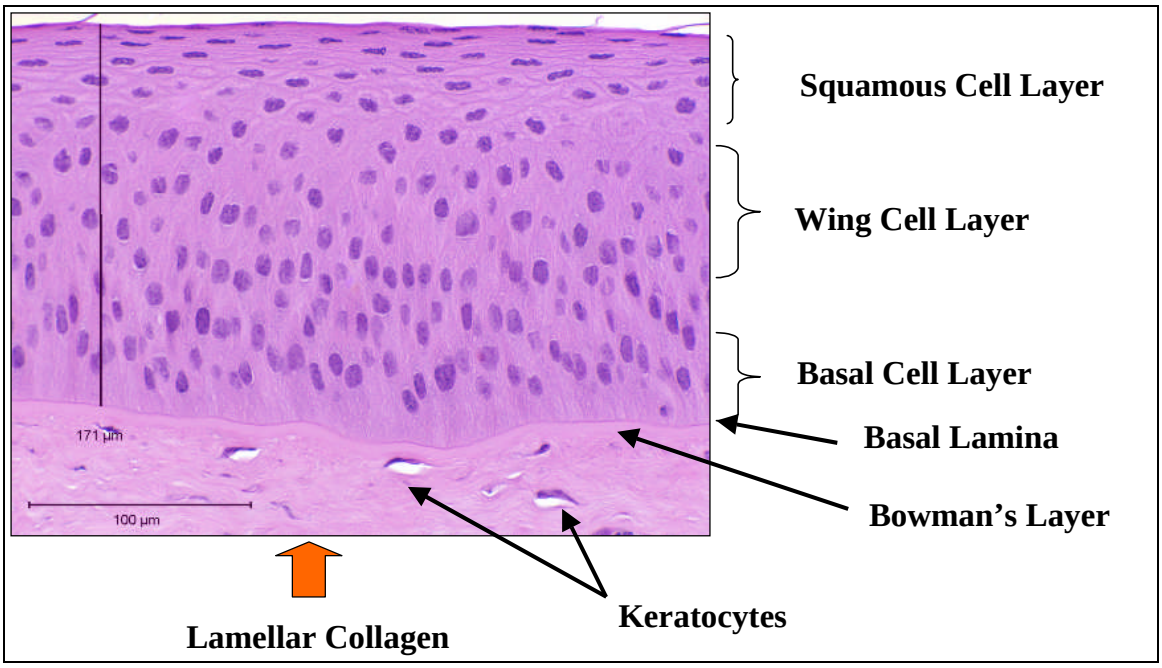


Figure 2. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (07/18/05) - Epithelium (magnification 237x)

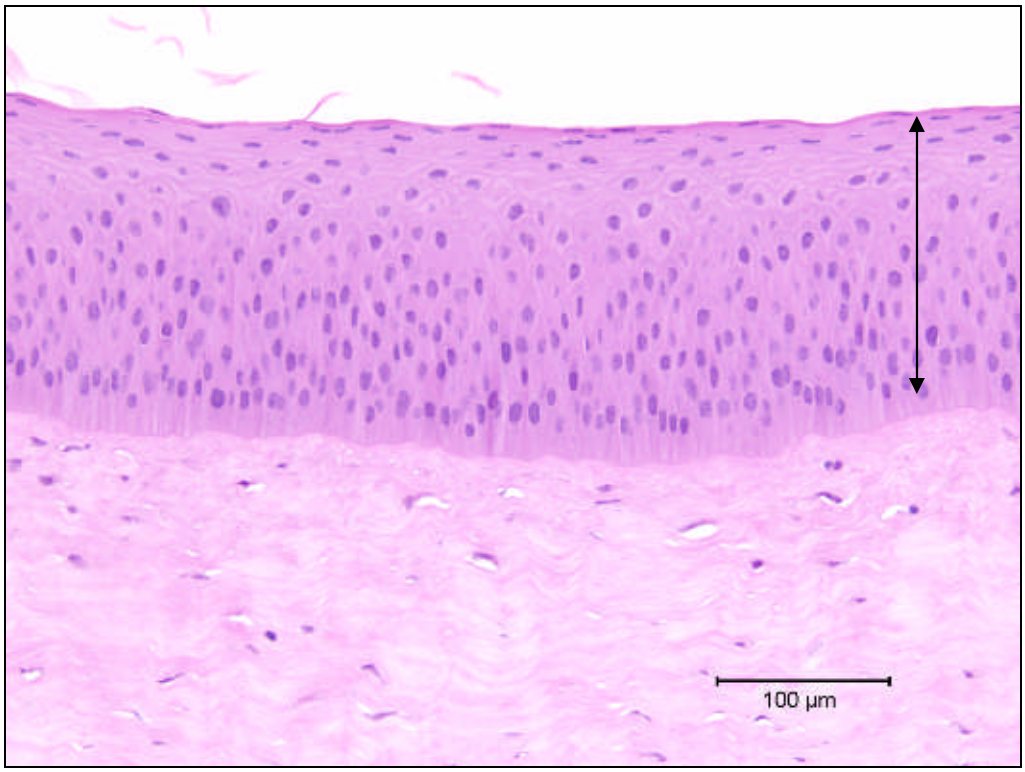


Figure 3. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (07/18/05) - Stroma directly below Bowman's Layer (magnification 475x)

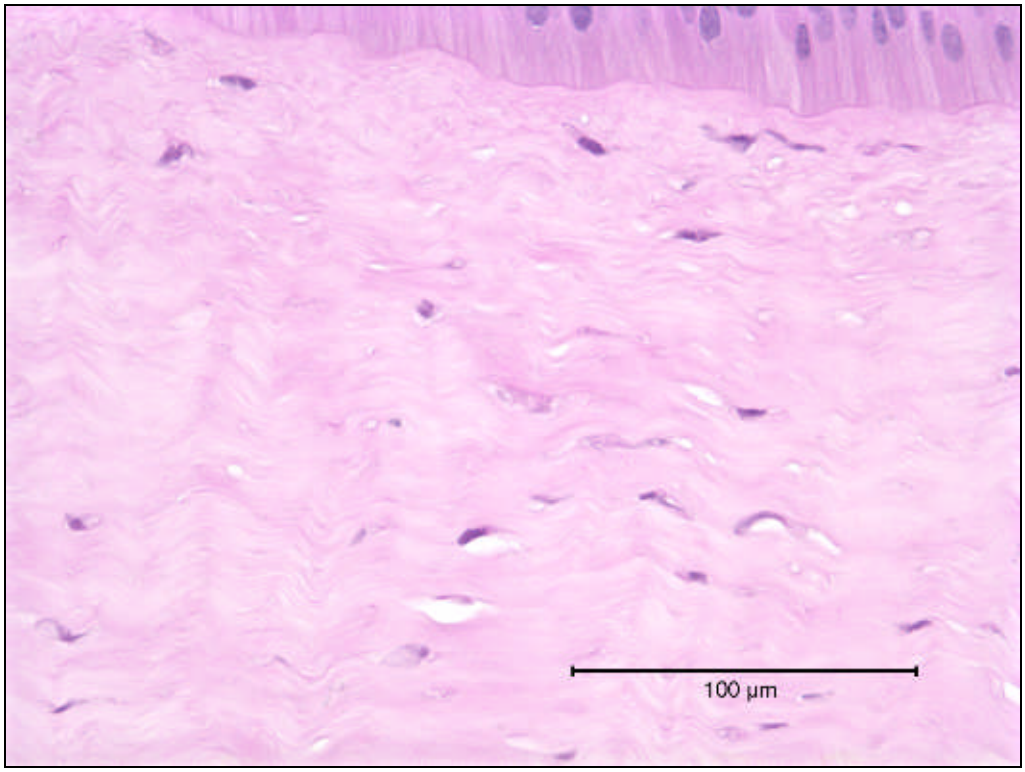


Figure 4. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (07/18/05) - Full thickness (magnification 48x)

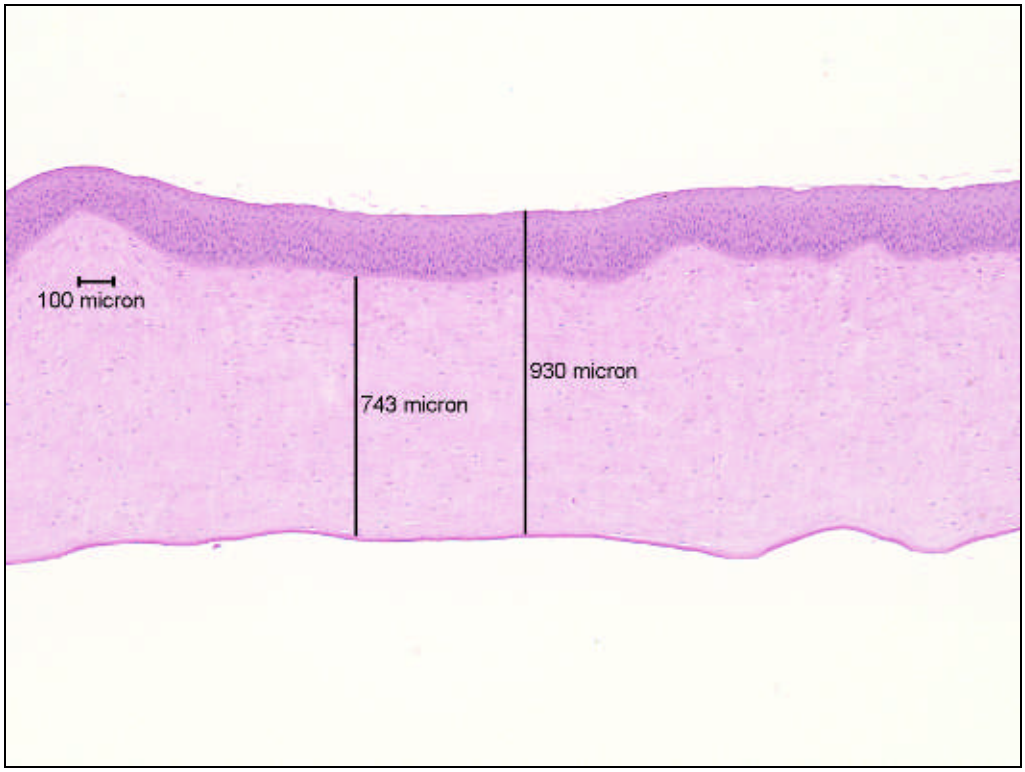


Figure 5. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/18/05) - Epithelium (probably not viable at the time of fixation) (magnification 237x)

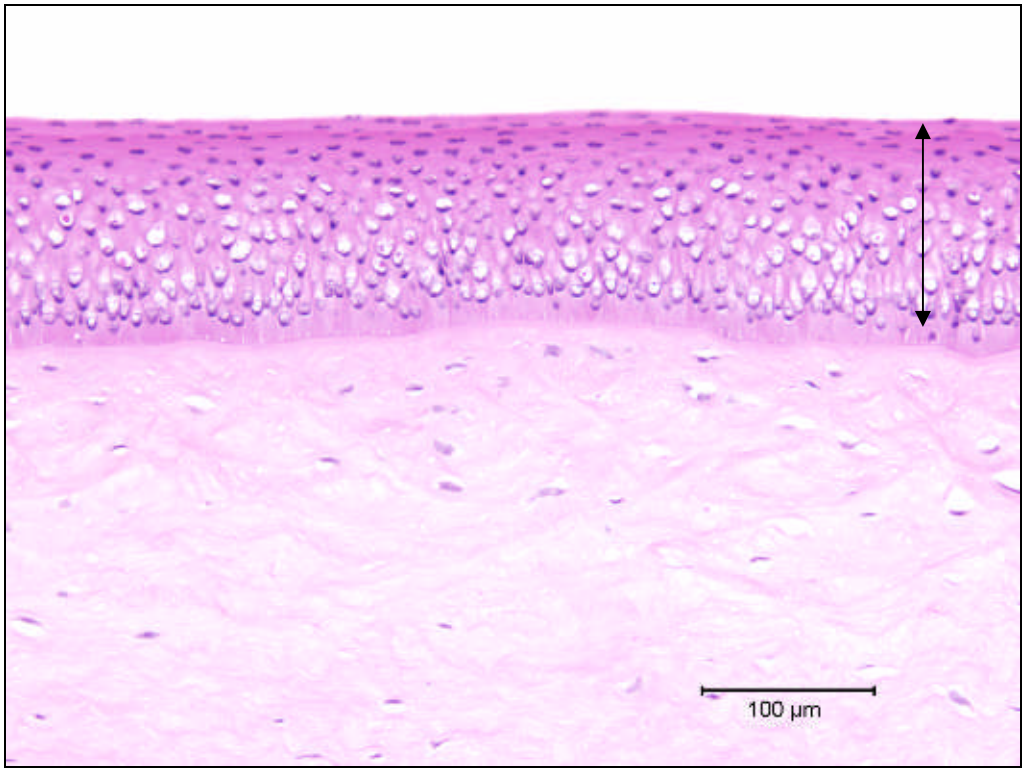


Figure 6. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/18/05) - Full thickness (magnification 48x)

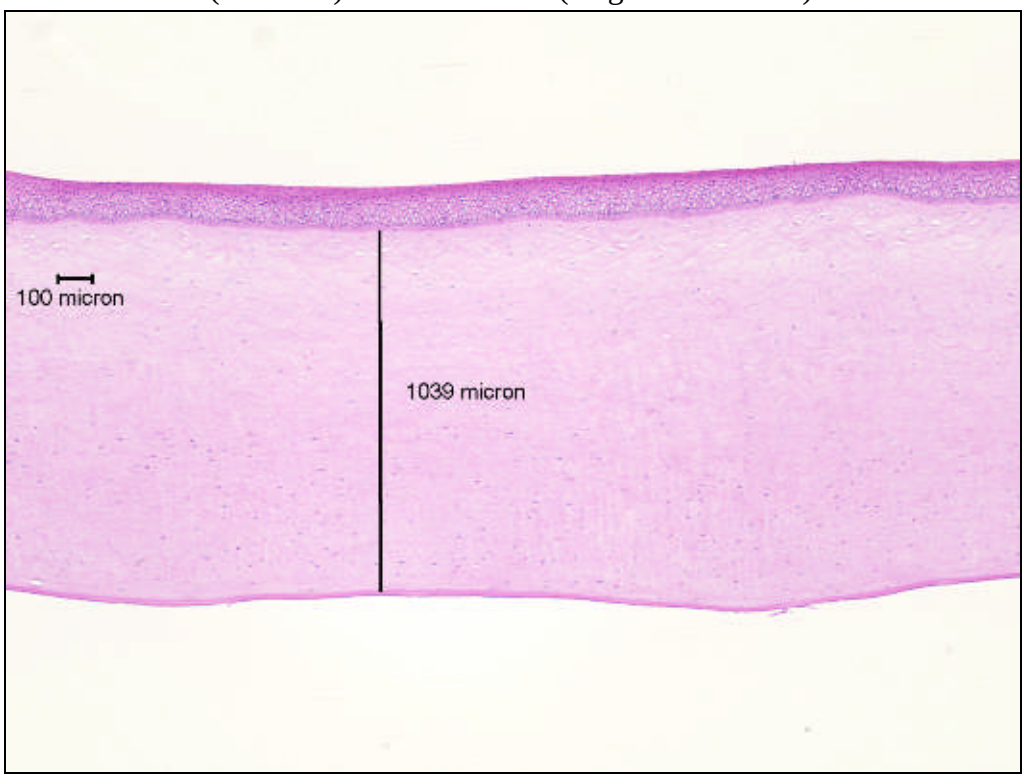


Figure 7. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/18/05) - Upper stroma showing hyperchromic staining in the zone directly below Bowman's Layer and the decrease in the density of viable keratocytes (magnification 237x)

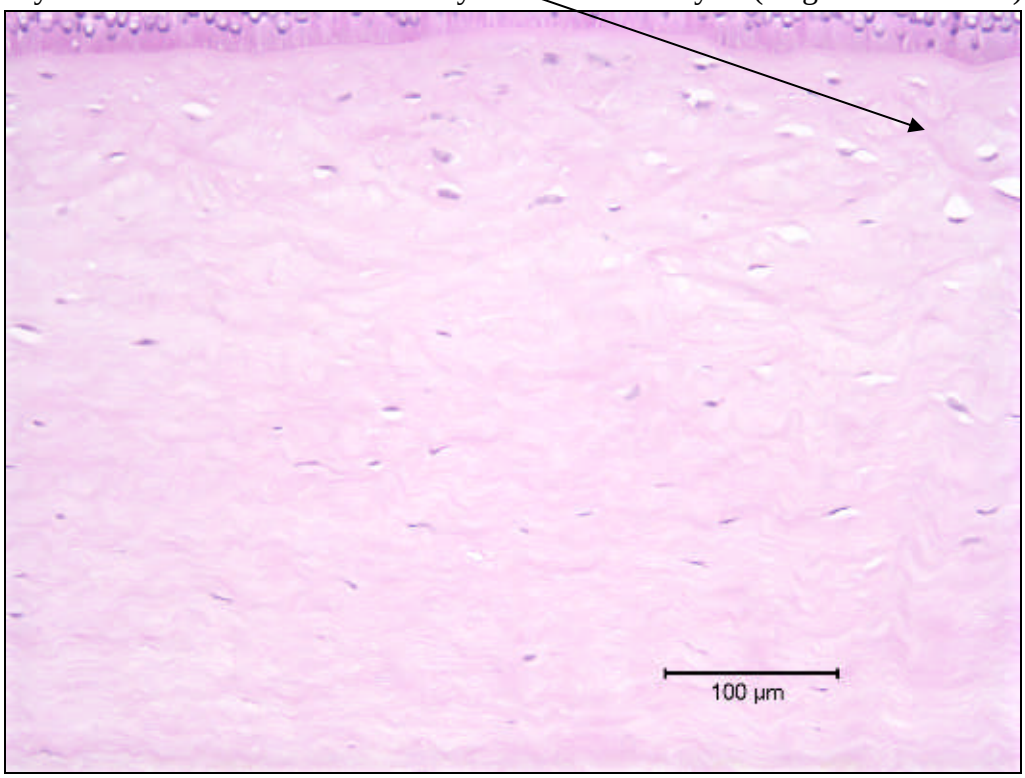


Figure 8. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/18/05) - Stroma at 20% depth showing moderate collagen matrix vacuolization and an increased frequency of keratocytes with abnormal chromatin condensation (magnification 475x)

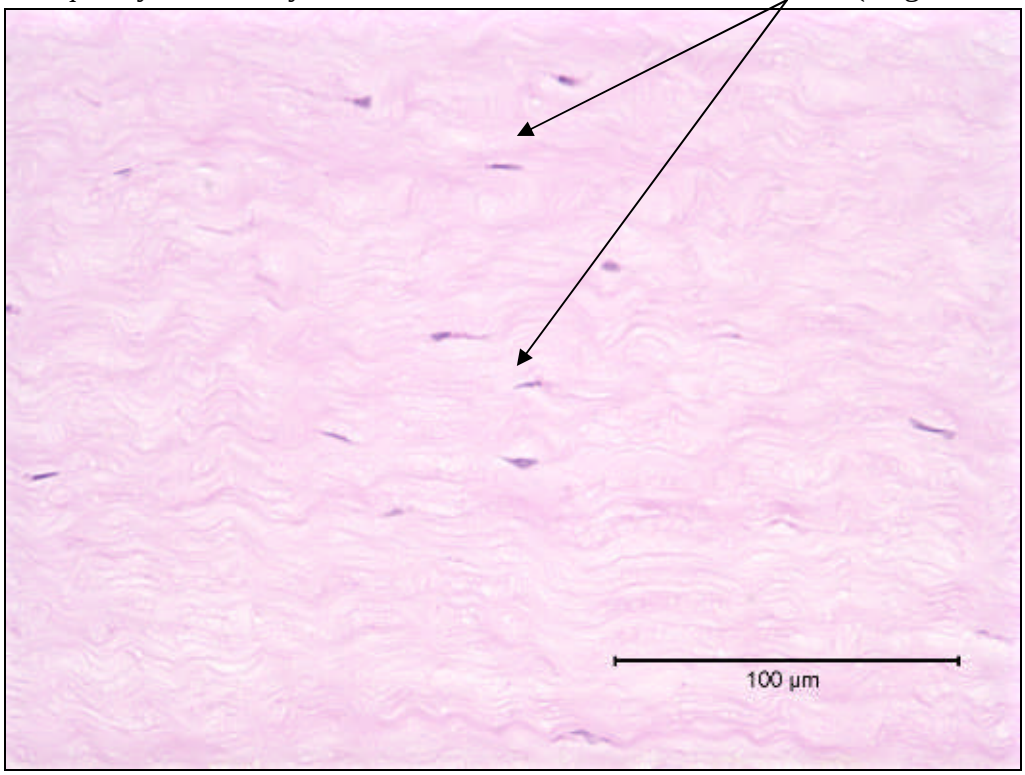


Figure 9. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/18/05) - Stroma below mid depth showing keratocyte with cytoplasmic eosinophilia (magnification 475x)

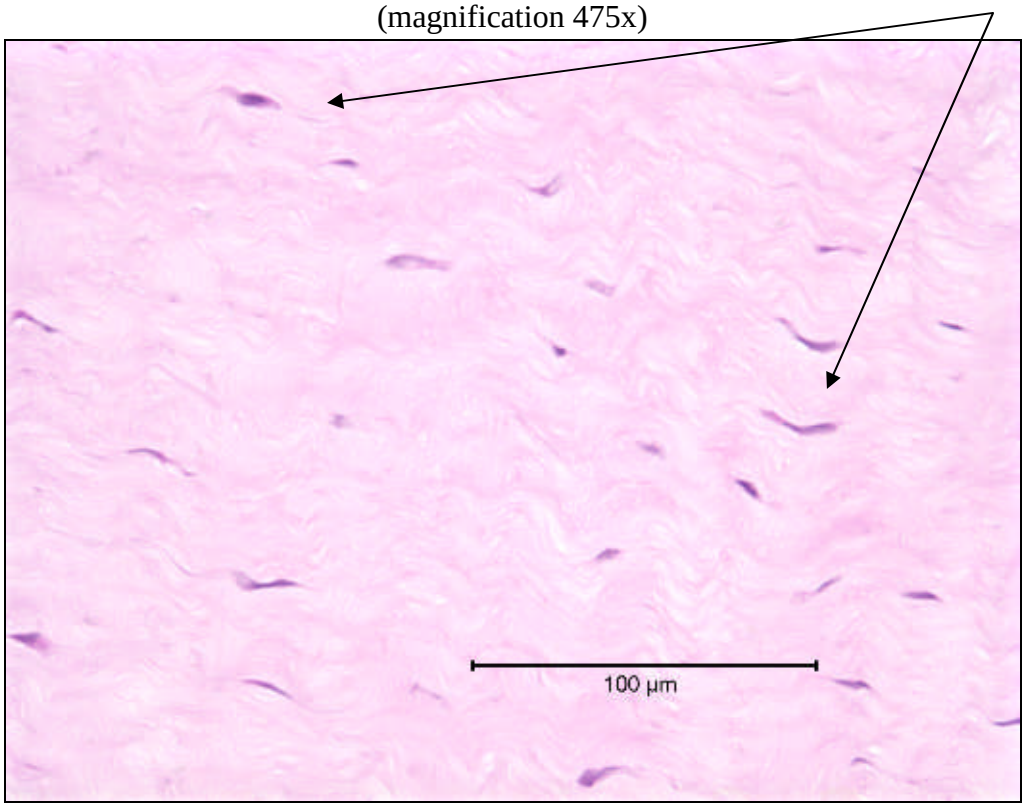


Figure 10. AV, neat, 3-minute exposure, 120-minute post-exposure (07/18/05) - Epithelial area (lost) (magnification 237x)

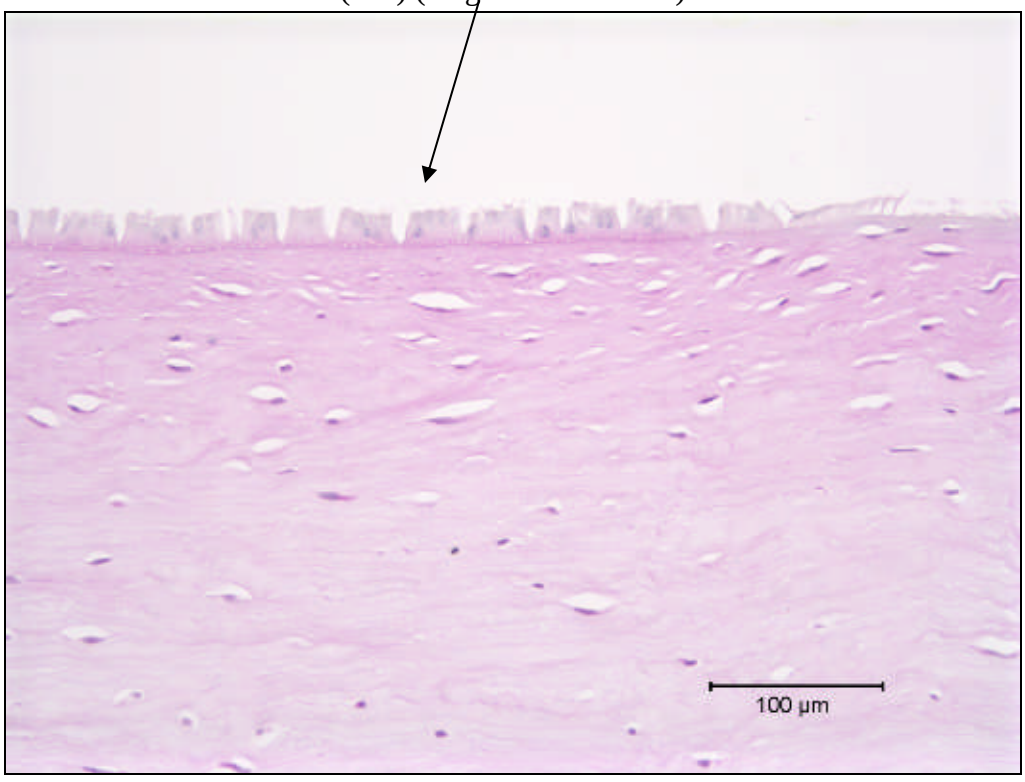


Figure 11. AV, neat, 3-minute exposure, 120-minute post-exposure (07/18/05) - Full thickness (magnification 48x)

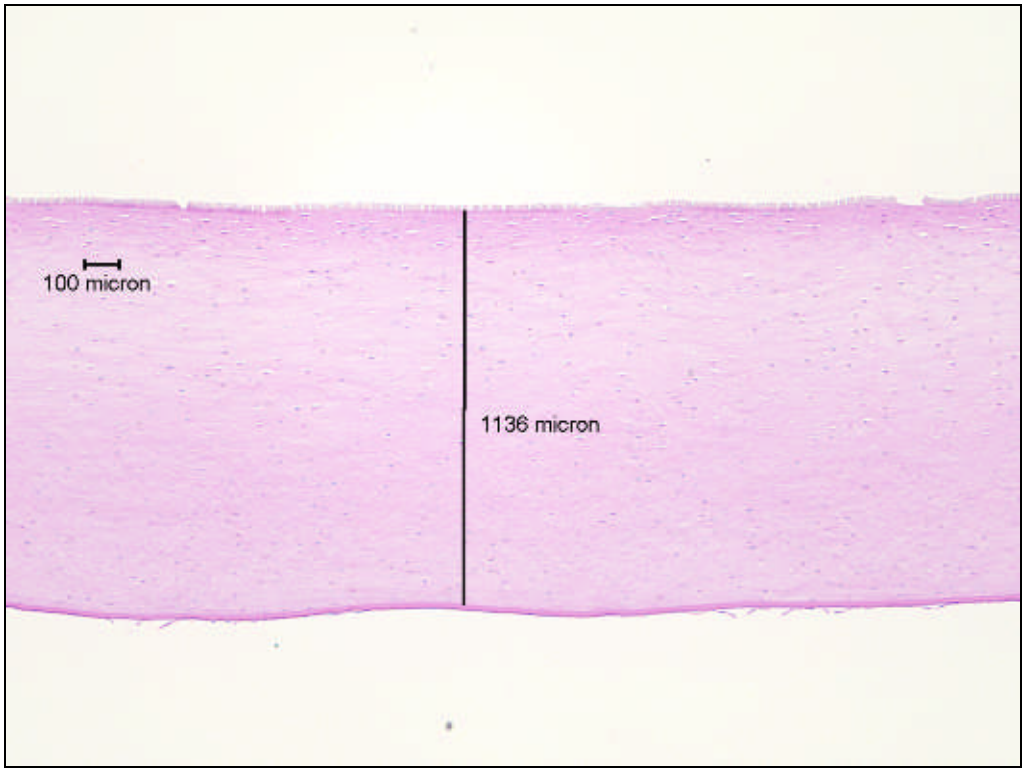


Figure 12. AV, neat, 3-minute exposure, 120-minute post-exposure (07/18/05) - Stroma at 20% depth showing moderate collagen matrix vacuolization and a marked increase in the frequency of keratocytes with nuclear granularization (magnification 475x)

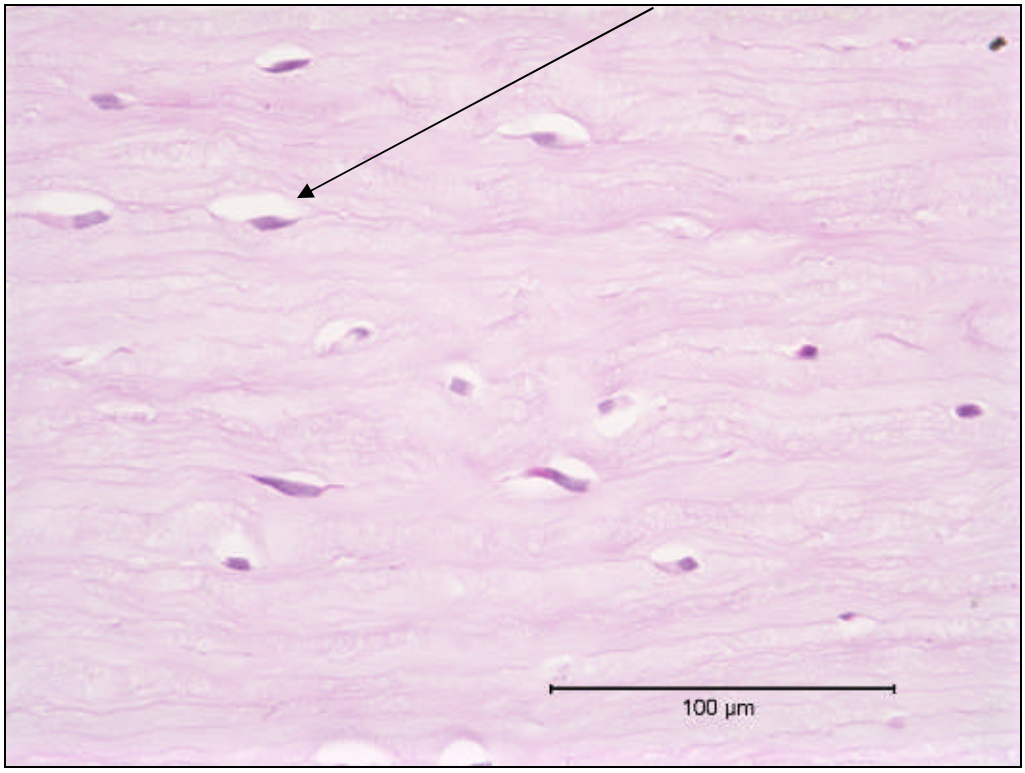


Figure 13. AV, neat, 3-minute exposure, 120-minute post-exposure (07/18/05) - Stroma above Descemet's Membrane showing a moderate increase in keratocytes with enlarged nuclei and cytoplasmic eosinophilia (magnification 475x)

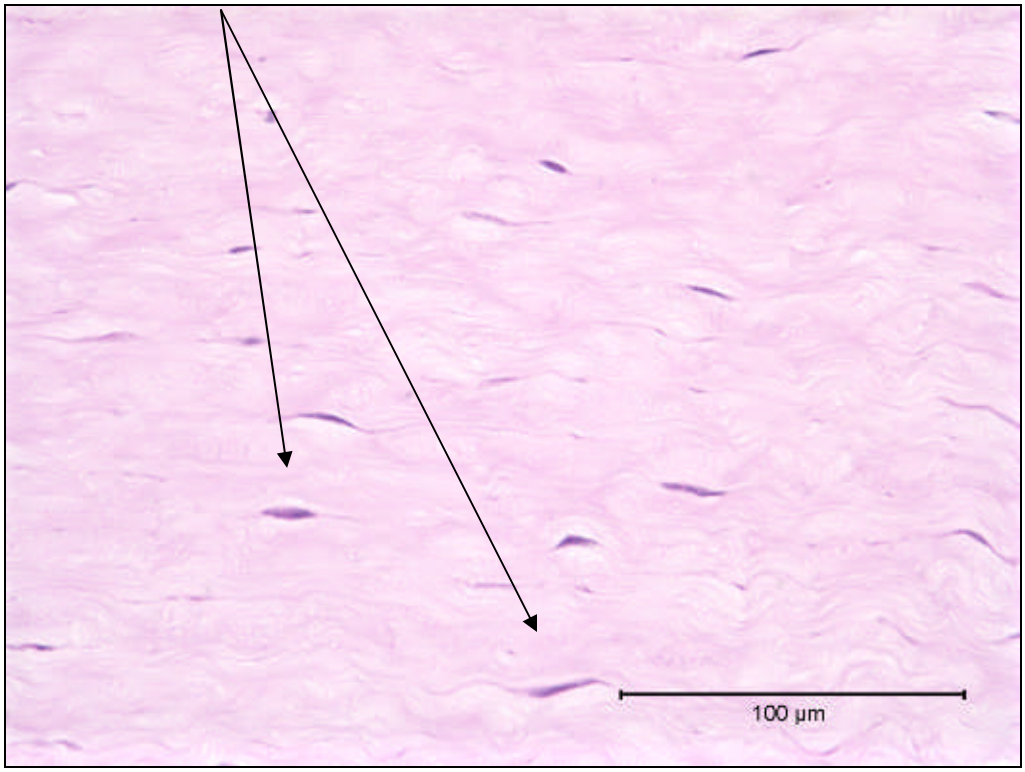


Figure 14. AV, neat, 10-minute exposure, 120-minute post-exposure (07/18/05) - Epithelium (lost) (magnification 237x)

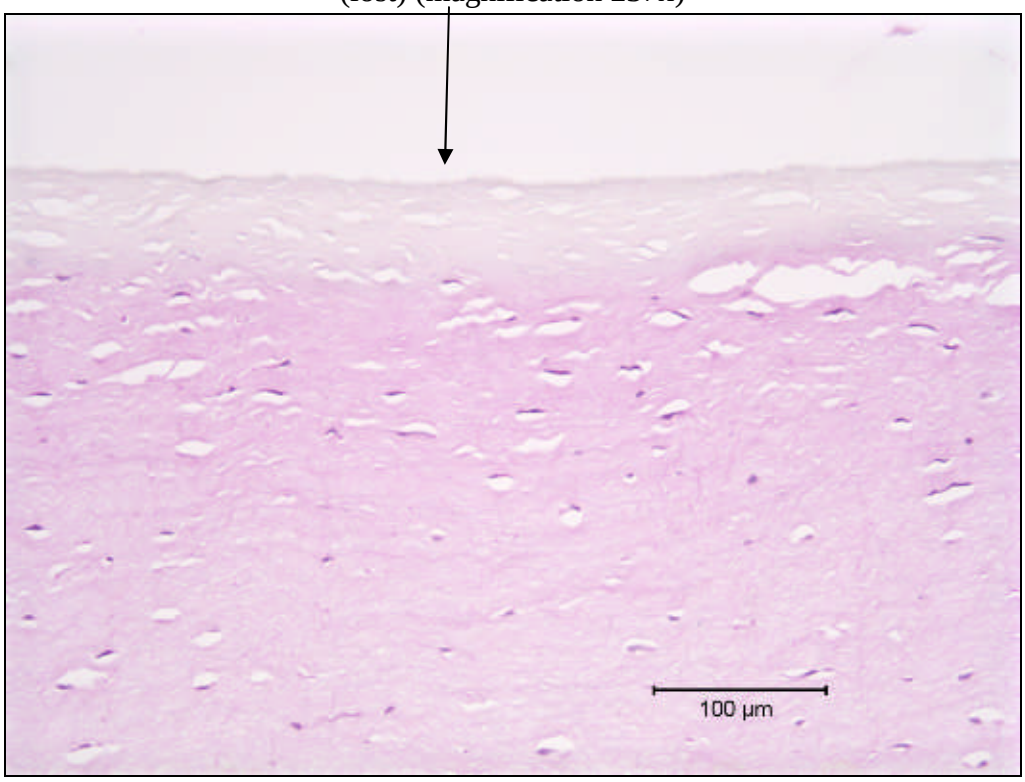


Figure 15. AV, neat, 10-minute exposure, 120-minute post-exposure (07/18/05) - Full thickness (magnification 48x)

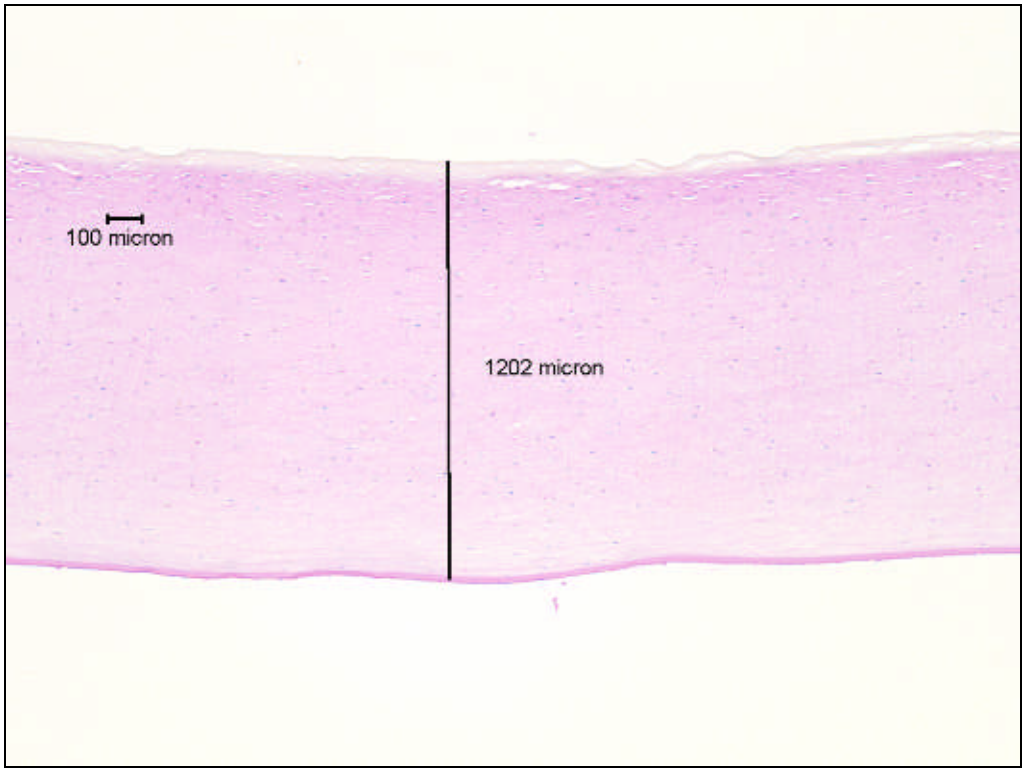


Figure 16. AV, neat, 10-minute exposure, 120-minute post-exposure (07/18/05) - Stroma at 20% depth showing only moderate vacuolization of the collagen matrix and degeneration of the keratocytes (magnification 475x)

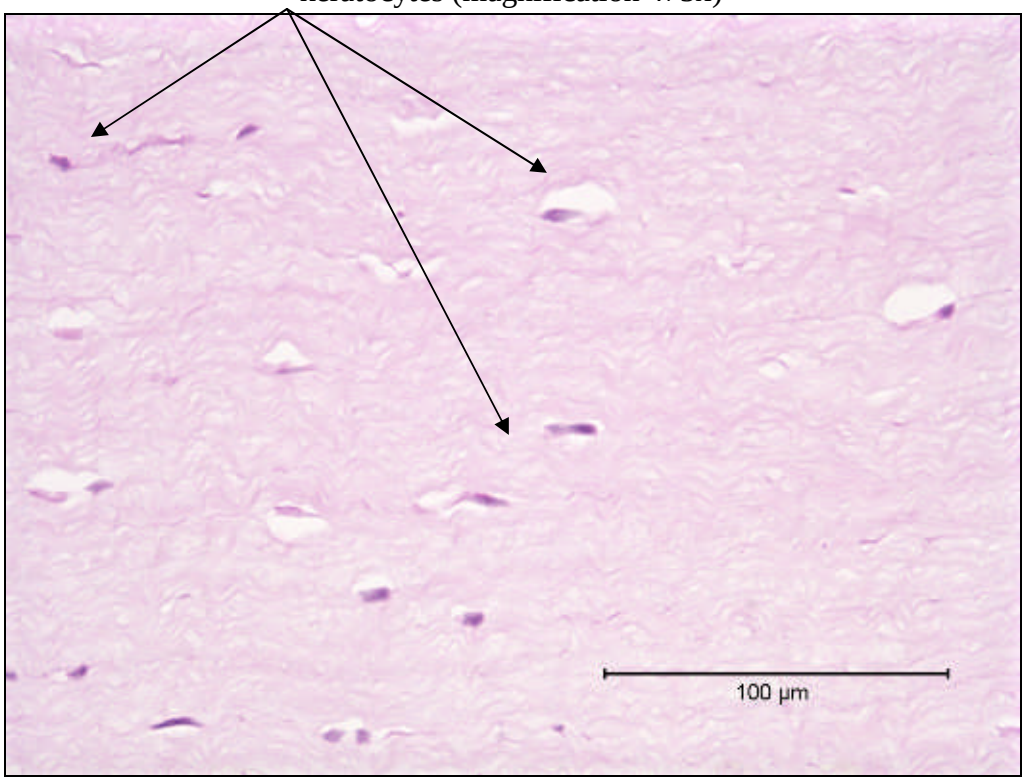


Figure 17. AV, neat, 10-minute exposure, 120-minute post-exposure (07/18/05) - Stroma above Descemet's Membrane showing marked collagen matrix vacuolization and marked damage to the keratocytes (magnification 475x)

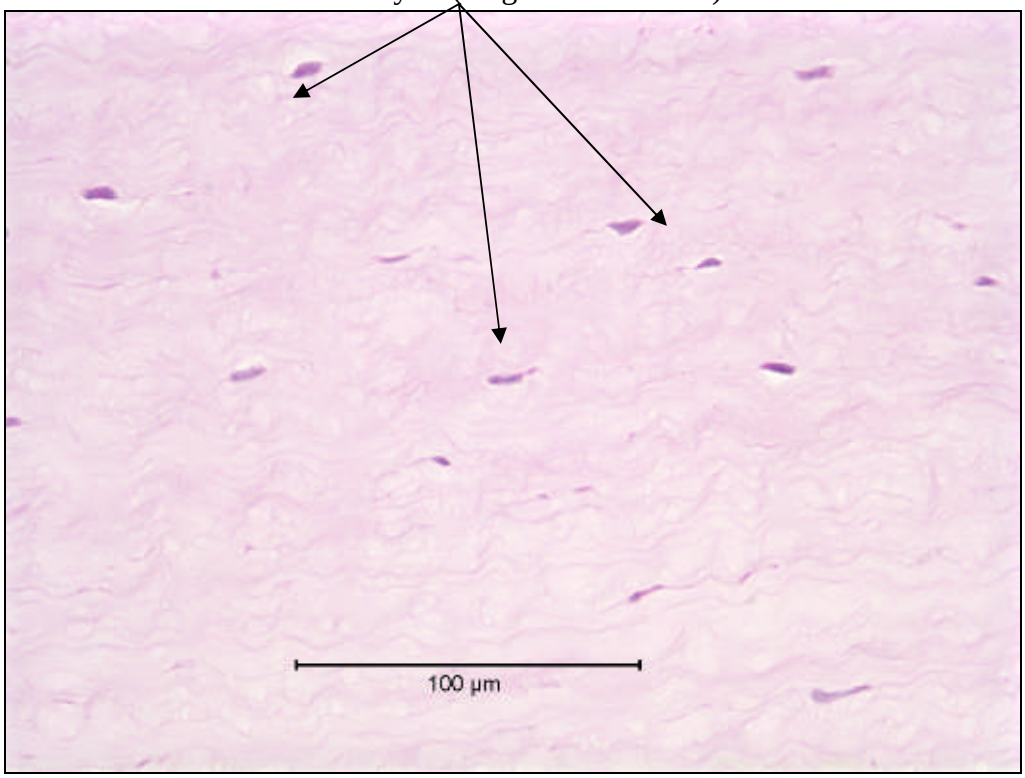


Figure 18. AW, neat, 3-minute exposure, 120-minute post-exposure (07/18/05) - Epithelium (largely lost) (magnification 237x)

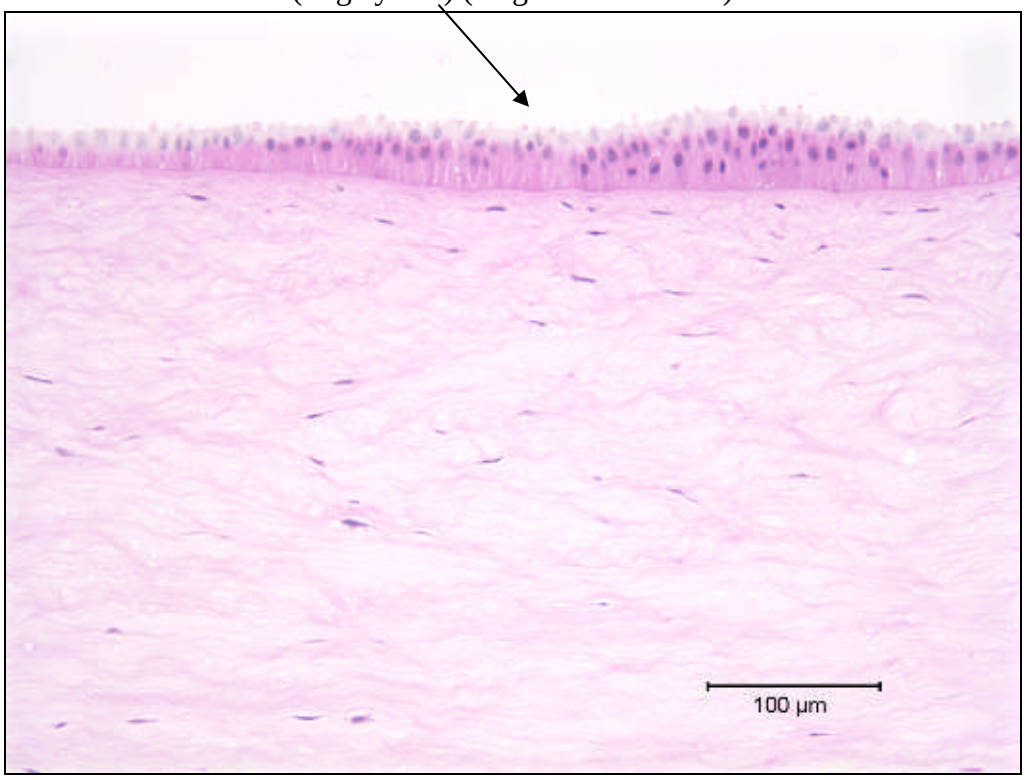


Figure 19. AW, neat, 3-minute exposure, 120-minute post-exposure (07/18/05) - Full thickness (magnification 48x)

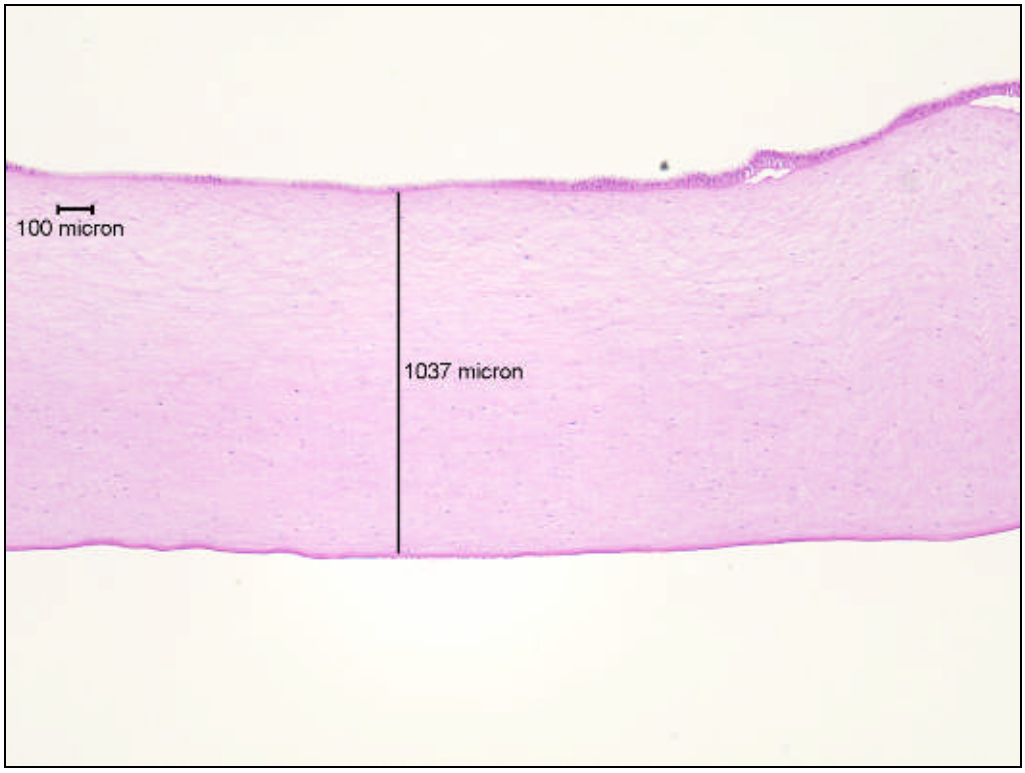


Figure 20. AW, neat, 3-minute exposure, 120-minute post-exposure (07/18/05) - Stroma at 20% depth showing marked collagen matrix vacuolization and a marked/moderate frequency of keratocytes with nuclear condensation and slight cytoplasmic eosinophilia (magnification 475x)

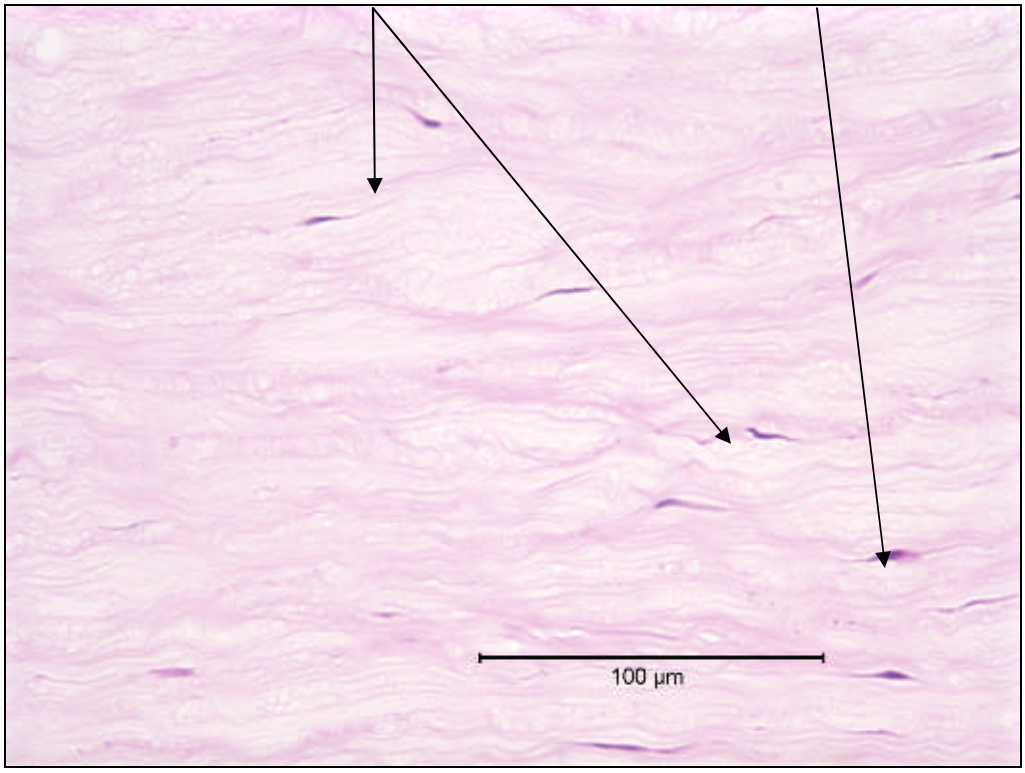


Figure 21. AW, neat, 3-minute exposure, 120-minute post-exposure (07/18/05) - Stroma at mid depth showing more moderate collagen matrix vacuolization and keratocytes with larger nuclei and cytoplasmic eosinophilia (magnification 475x)

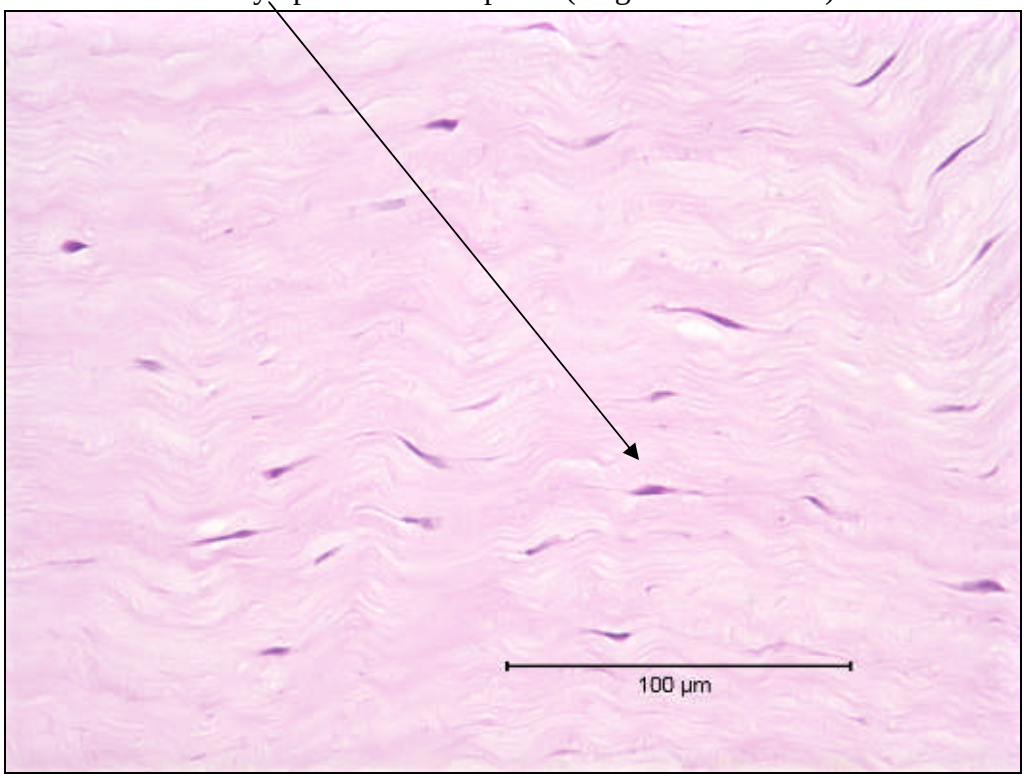


Figure 22. AW, neat, 10-minute exposure, 120-minute post-exposure (07/18/05)(cornea #20) - Epithelium (largely lost) (magnification 237x)

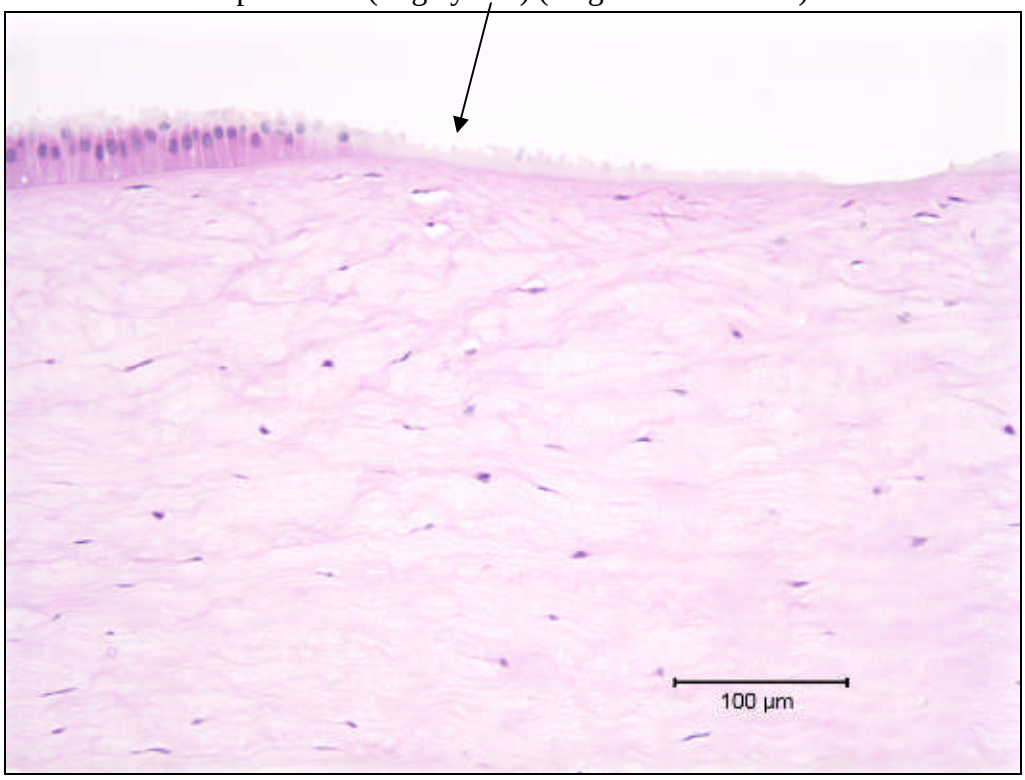


Figure 23. AW, neat, 10-minute exposure, 120-minute post-exposure (07/18/05)(cornea #23) - Epithelium (more retained on this cornea) (magnification 237x)

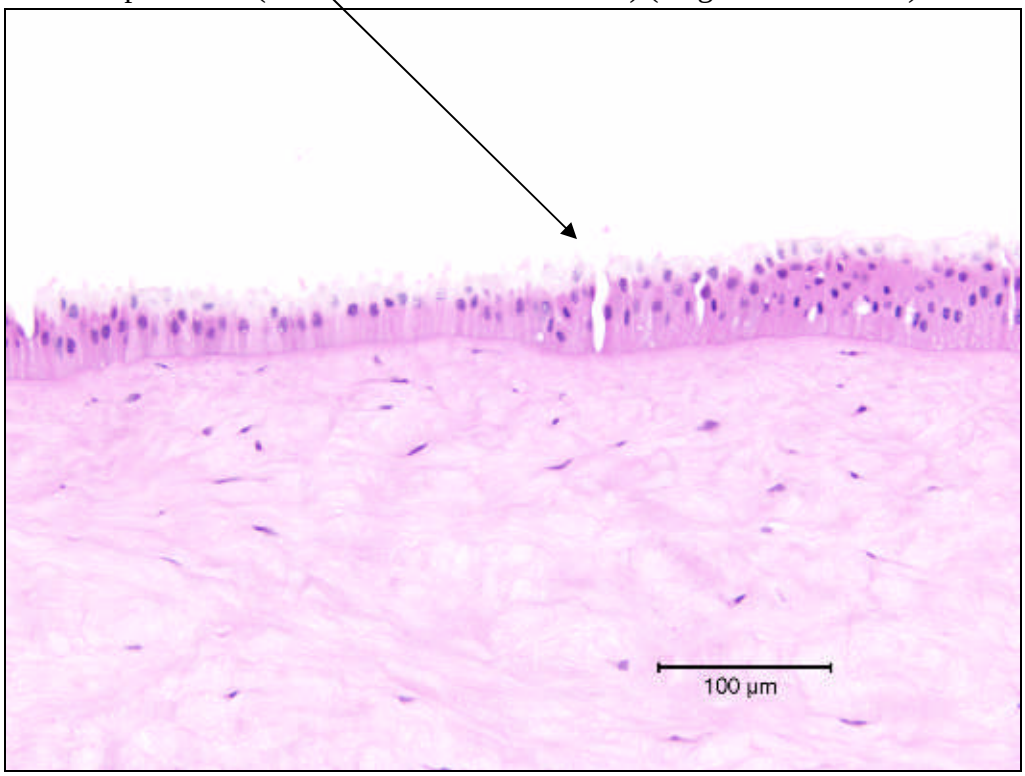


Figure 24. AW, neat, 10-minute exposure, 120-minute post-exposure (07/18/05)(cornea #20) - Full thickness (magnification 48x)

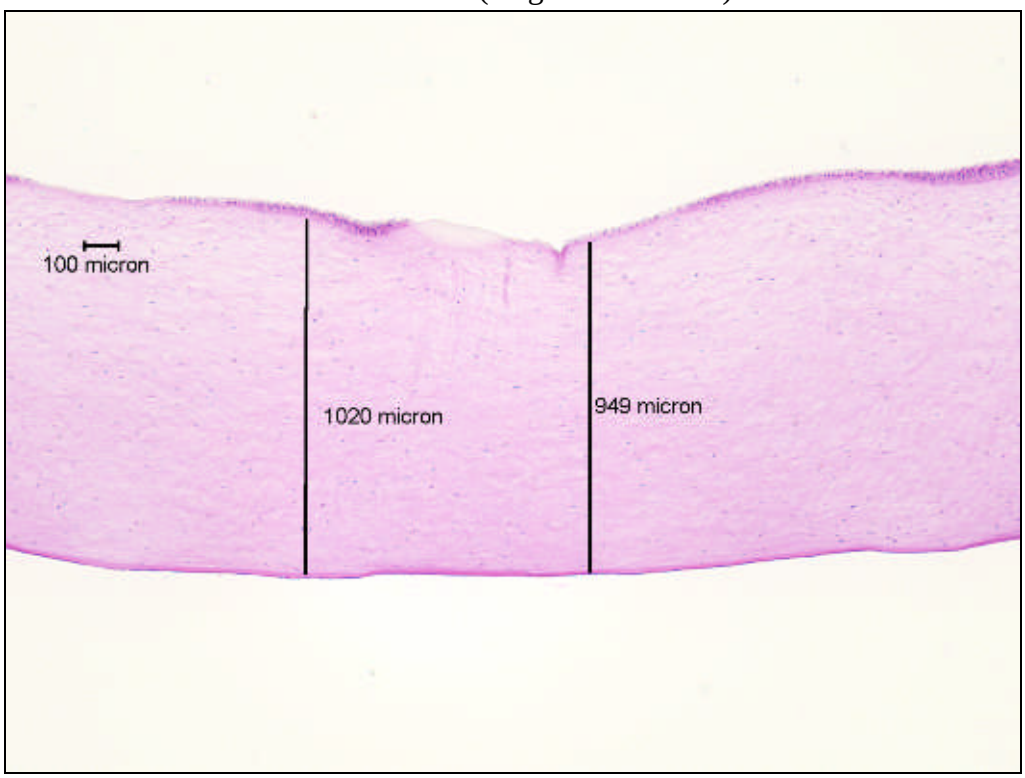


Figure 25. AW, neat, 10-minute exposure, 120-minute post-exposure (07/18/05)(cornea #20) - Stroma at 20% depth showing marked collagen matrix vacuolization and a marked/moderate frequency of keratocytes with nuclear condensation and slight cytoplasmic eosinophilia (magnification 475x)

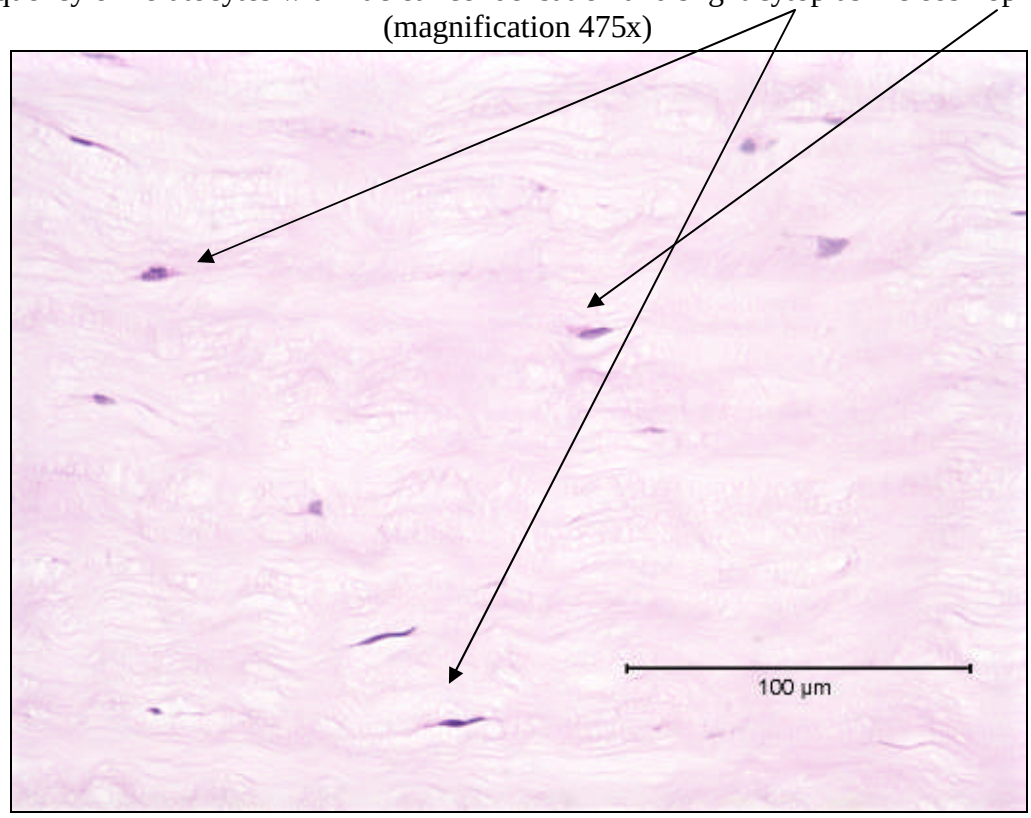
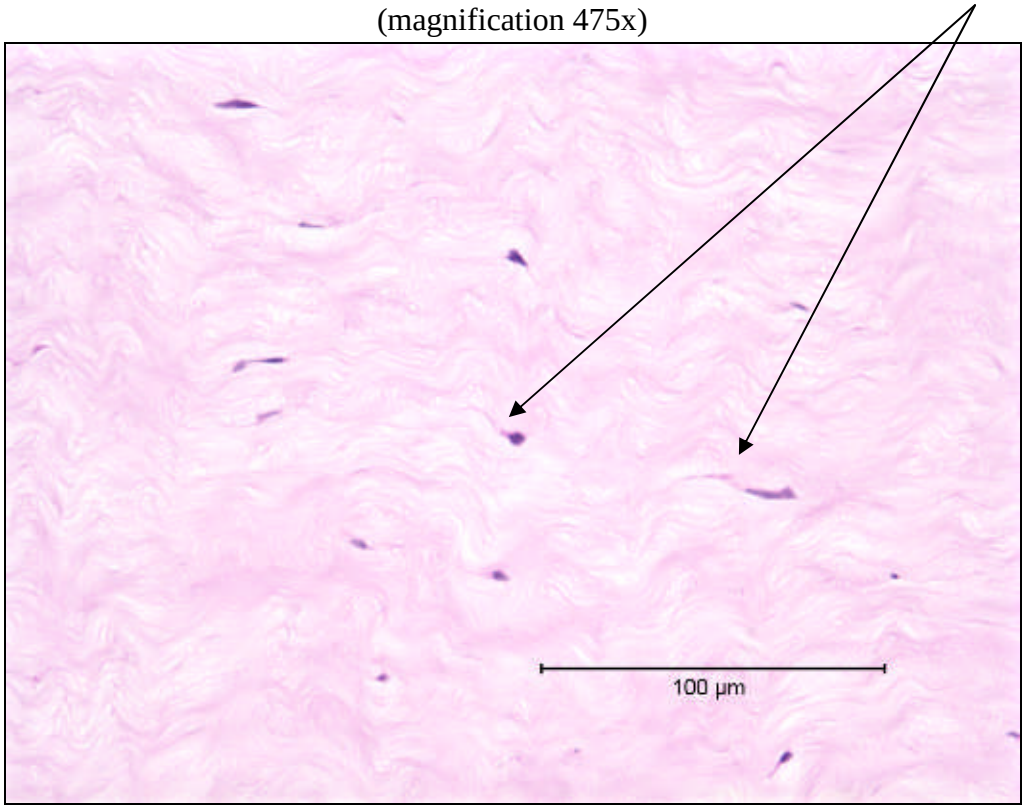


Figure 26. AW, neat, 10-minute exposure, 120-minute post-exposure (07/18/05)(cornea #20) - Deep stroma showing keratocytes with nuclear enlargement and cytoplasmic eosinophilia (magnification 475x)



APPENDIX A

APPENDIX B

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

~~TA #~~	~~CORNEA #~~	~~INITIAL~~	~~FINAL~~	~~CHANGE~~	~~CORRECTED~~	~~AVG~~	~~STDEV~~
05AD93	8	3	10	7	5.7		
Neat	9	3	11	8	6.7		
3 minutes	10	3	14	11	9.7	7.3	2.1
05AD93	11	3	9	6	4.7		
Neat	12	4	10	6	4.7		
10 minutes	13	3	16	13	11.7	7.0	4.0
05AD94	14	3	31	28	26.7		
Neat	16	3	33	30	28.7		
3 minutes	17	3	31	28	26.7	27.3	1.2
05AD94	18	3	47	44	42.7		
Neat	19	3	50	47	45.7		
10 minutes	20	3	58	55	53.7	47.3	5.7
05AD95	22	5	13	8	6.7		
Neat	25	3	10	7	5.7		
3 minutes	26	5	11	6	4.7	5.7	1.0
05AD95	27	4	9	5	3.7		
Neat	28	4	9	5	3.7		
10 minutes	29	6	17	11	9.7	5.7	3.5
05AD96	30	4	12	8	6.7		
Neat	32	4	12	8	6.7		
3 minutes	33	4	11	7	5.7	6.3	0.6
05AD96	34	4	8	4	2.7		
Neat	35	4	8	4	2.7		
10 minutes	36	4	9	5	3.7	3.0	0.6
05AD97	37	4	23	19	17.7		
Neat	38	5	30	25	23.7		
3 minutes	39	4	24	20	18.7	20.0	3.2
05AD97	40	4	56	52	50.7		
Neat	42	5	55	50	48.7		
10 minutes	44	5	63	58	56.7	52.0	4.2
05AD98	45	3	30	27	25.7		
Neat	46	3	32	29	27.7		
3 minutes	47	4	32	28	26.7	26.7	1.0
05AD98	48	5	102	97	95.7		
Neat	49	4	110	106	104.7		
10 minutes	51	4	107	103	101.7	100.7	4.6
Neg. Control	1	3	4	1	NA		
Sterile, DI water	2	3	5	2	NA		
10 minutes	4	3	4	1	NA	1.3	
Pos. Control	5	3	35	32	30.7		
Ethanol	6	3	29	26	24.7		
10 minutes	7	5	31	26	24.7	26.7	3.5
	*24	2					
	*41	6					

Initial corneal opacity average: 4

* - Corneas not used in this assay, but used to find initial opacity average.
NA - Not Applicable

PERMEABILITY SCORE

Neg. Control
Sterile, DI water
10 minutes

Cornea #	OD490
1	0.004
2	0.005
4	0.005
Avg.	0.005

Pos. Control
Ethanol
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
5	0.805	1	0.800
6	0.886	1	0.881
7	1.233	1	1.228
		Avg. =	0.970
		STDEV =	0.227

05AD93
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
8	0.531	5	2.650
9	0.577	5	2.880
10	0.515	5	2.570
		Avg. =	2.700
		STDEV =	0.161

05AD93
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
11	1.012	5	5.055
12	1.176	5	5.875
13	0.929	5	4.640
		Avg. =	5.190
		STDEV =	0.628

05AD94
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
14	0.440	5	2.195
16	0.815	5	4.070
17	0.540	5	2.695
		Avg. =	2.987
		STDEV =	0.971

05AD94
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
18	0.944	5	4.715
19	0.919	5	4.590
20	0.889	5	4.440
		Avg. =	4.582
		STDEV =	0.138

05AD95
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
22	0.430	5	2.145
25	0.398	5	1.985
26	0.411	5	2.050
		Avg. =	2.060
		STDEV =	0.080

05AD95
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
27	0.973	5	4.860
28	1.182	5	5.905
29	0.814	5	4.065
		Avg. =	4.944
		STDEV =	0.923

05AD96
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
30	0.513	5	2.560
32	0.611	5	3.050
33	0.618	5	3.085
		Avg. =	2.899
		STDEV =	0.294

05AD96
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
34	1.175	5	5.870
35	1.153	5	5.760
36	0.977	5	4.880
		Avg. =	5.504
		STDEV =	0.543

05AD97
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
37	0.527	5	2.630
38	0.624	5	3.115
39	0.633	5	3.160
		Avg. =	2.969
		STDEV =	0.294

05AD97
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
40	0.858	5	4.285
42	0.894	5	4.465
44	1.057	5	5.280
		Avg. =	4.677
		STDEV =	0.530

05AD98
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
45	0.549	5	2.740
46	0.452	5	2.255
47	0.686	5	3.425
		Avg. =	2.807
		STDEV =	0.588

05AD98
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
48	1.249	5	6.240
49	1.294	5	6.465
51	1.107	5	5.530
		Avg. =	6.079
		STDEV =	0.488

IN VITRO SCORE

In Vitro Score = Mean Opacity Value + (15 x Mean OD490)

Test Article	Concentration	Exposure Period	Mean Opacity	Mean OD490	In vitro Score
05AD93	Neat	3 minutes	7.3	2.700	47.8
05AD93	Neat	10 minutes	7.0	5.190	84.9
05AD94	Neat	3 minutes	27.3	2.987	72.1
05AD94	Neat	10 minutes	47.3	4.582	116.1
05AD95	Neat	3 minutes	5.7	2.060	36.6
05AD95	Neat	10 minutes	5.7	4.944	79.8
05AD96	Neat	3 minutes	6.3	2.899	49.8
05AD96	Neat	10 minutes	3.0	5.504	85.6
05AD97	Neat	3 minutes	20.0	2.969	64.5
05AD97	Neat	10 minutes	52.0	4.677	122.2
05AD98	Neat	3 minutes	26.7	2.807	68.8
05AD98	Neat	10 minutes	100.7	6.079	191.8
Ethanol	Neat	10 minutes	26.7	0.970	41.2

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

TA #	CORNEA #	INITIAL	FINAL	CHANGE	CORRECTED	AVG	STDEV
05AD98	9	3	42	39	35.7		
Neat	10	5	38	33	29.7		
3 minutes	11	4	41	37	33.7	33.0	3.1
05AD98	12	4	101	97	93.7		
Neat	13	3	113	110	106.7		
10 minutes	14	2	93	91	87.7	96.0	9.7
05AD99	15	3	10	7	3.7		
Neat	17	3	11	8	4.7		
3 minutes	18	3	10	7	3.7	4.0	0.6
05AD99	20	3	7	4	0.7		
Neat	22	3	9	6	2.7		
10 minutes	23	4	12	8	4.7	2.7	2.0
05AE00	24	2	48	46	42.7		
Neat	25	5	54	49	45.7		
3 minutes	27	4	49	45	41.7	43.3	2.1
05AE00	28	4	121	117	113.7		
Neat	29	4	141	137	133.7		
10 minutes	31	5	120	115	111.7	119.7	12.2
Neg. Control	1	4	7	3	NA		
Sterile, DI water	2	4	4	0	NA		
60 minutes	5	5	12	7	NA	3.3	
Pos. Control	6	3	36	33	29.7		
Ethanol	7	5	41	36	32.7		
10 minutes	8	4	39	35	31.7	31.3	1.5
	*21	5					
	*30	6					
	*34	5					
	*35	4					
	*36	5					
	*37	5					
	*38	4					
	*39	6					
	*40	3					
	*41	4					
	*42	7					
	*43	7					
	*45	5					

Initial corneal opacity average: 4

* - Corneas not used in this assay, but used to find initial opacity average.
NA - Not Applicable

PERMEABILITY SCORE

Neg. Control
Sterile, DI water
60 minutes

Cornea #	OD490
1	0.002
2	0.004
5	0.002
Avg.	0.003

Pos. Control
Ethanol
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
6	1.331	1	1.328
7	1.210	1	1.207
8	1.059	1	1.056
		Avg. =	1.197
		STDEV=	0.136

05AD98
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
9	0.798	5	3.987
10	0.811	5	4.052
11	0.518	5	2.587
		Avg. =	3.542
		STDEV=	0.828

05AD98
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
12	1.137	5	5.682
13	1.099	5	5.492
14	1.179	5	5.892
		Avg. =	5.689
		STDEV=	0.200

05AD99
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
15	0.347	5	1.732
17	0.337	5	1.682
18	0.343	5	1.712
		Avg. =	1.709
		STDEV=	0.025

05AD99
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
20	0.661	5	3.302
22	0.696	5	3.477
23	1.308	1	1.305
		Avg. =	2.695
		STDEV=	1.207

05AE00
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
24	0.402	5	2.007
25	0.403	5	2.012
27	0.651	5	3.252
		Avg. =	2.424
		STDEV=	0.717

05AE00
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
28	0.990	5	4.947
29	1.069	5	5.342
31	0.929	5	4.642
		Avg. =	4.977
		STDEV=	0.351

IN VITRO SCORE

In Vitro Score = Mean Opacity Value + (15 x Mean OD490)

Test Article	Concentration	Exposure Period	Mean Opacity	Mean OD490	In vitro Score
05AD98	Neat	3 minutes	33.0	3.542	86.1
05AD98	Neat	10 minutes	96.0	5.689	181.3
05AD99	Neat	3 minutes	4.0	1.709	29.6
05AD99	Neat	10 minutes	2.7	2.695	43.1
05AE00	Neat	3 minutes	43.3	2.424	79.7
05AE00	Neat	10 minutes	119.7	4.977	194.3
Ethanol	Neat	10 minutes	31.3	1.197	49.3